THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Cellular Uptake of Amyloid Forming Proteins Related to Neurodegenerative Disease

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Cover:
The illustration shows a mammalian cell internalising monomeric and fibrillar forms of amyloidogenic proteins by endocytosis, resulting in intracellular accumulation. Different types of clathrin-independent endocytic mechanisms are depicted, as well as uptake via interaction with cell surface proteoglycans.

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ABSTRACT

Aggregation and deposition of disease-associated protein is a pathological hallmark of several human disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD). These diseases are characterized by the formation of amyloid-β (Aβ) and α-synuclein (α-syn) amyloid fibrils, in extracellular and intracellular locations, respectively. Prior to extracellular deposition of Aβ into plaques, Aβ also accumulates within neurons, but the molecular and cellular mechanisms contributing to uptake are not fully understood. Moreover, exact links between disease onset and progression are missing, hindering the development of new disease-modifying therapies.

This Thesis describes my research to elucidate how chemical and physical characteristics of Aβ and α-syn, and their ensuing aggregates, influence their cellular uptake. This is important as the endolysosomal system has been implicated as a potential site for onset and progression of disease pathology. Focusing on Aβ uptake I demonstrate that the most aggregation-prone and neurotoxic variant Aβ(1-42) is endocytosed twice as efficiently as Aβ(1-40). I show that the uptake of both variants occurs via clathrin- and dynamin-independent endocytosis, but my work also points to a mechanistic difference; Aβ(1-42) is for example more sensitive to inhibitors of action polymerisation. Further, in studies of Aβ(1-42), I demonstrate that uptake is regulated by small Rho GTPases and highly sensitive to changes in membrane tension, but apparently not via GRAF1-regulated CLIC/GEECs, suggesting the involvement of yet unidentified molecular players.

I also show how uptake of pre-formed α-syn fibrils is inversely related to fibril length, and correlates to reductions in metabolic activity, pointing to an important role of cellular uptake and endolysosomal accumulation in toxicity. Lastly, I demonstrate that both monomeric Aβ and fibrillar α-syn are dependent on cell surface proteoglycans for uptake. Importantly, I show that for Aβ this dependency builds up over time, suggesting that local peptide aggregation at the cell surface could precede uptake.

Altogether, this Thesis contribute new molecular and mechanistic insights into how cellular uptake contributes to intraneuronal accumulation of amyloidogenic proteins relevant in neurodegenerative disease.

Keywords: Alzheimer's disease, amyloid fibril, amyloid-β, Aβ(1-40), Aβ(1-42), SH-SY5Y, α-synuclein, confocal microscopy, flow cytometry, fluorescence

LIST OF PUBLICATIONS

This Thesis is based on the work contained in the following research papers[1]:

I. Endocytic uptake of monomeric amyloid-β peptides is clathrin- and dynamin-independent and results in selective accumulation of Aβ(1–42) compared to Aβ(1–40)
Emelie Wesén, Gavin D. M. Jeffries, Maria Matson Dzebo, Elin K. Esbjörner.
Scientific Reports, 7(1):2021, (2017). doi: 10.1038/s41598-017-02227-9

II. Correlation between cellular uptake and cytotoxicity of fragmented α-synuclein amyloid fibrils suggests intracellular basis for toxicity
Xiaolu Zhang‡, Emelie Wesén‡, Ranjeet Kumar, David Bernson, Audrey Gallud, Alexandra Paul, Pernilla Wittung-Stafshede, Elin K. Esbjörner.
ACS Chemical Neuroscience, (2020). doi: 10.1021/acschemneuro.9b00562
‡ Both authors contributed equally to this work.

III. Cell surface proteoglycan-mediated uptake and accumulation of the Alzheimer's disease peptide Aβ(1–42)
Emelie Wesén, Audrey Gallud, Alexandra Paul, David J. Lindberg, Per Malmberg, Elin K. Esbjörner.
BBA Biomembranes, 1860(11), (2018), 2204-2214.
doi: 10.1016/j.bbamem.2018.08.010

IV. Role of membrane tension and Rho GTPases in endocytosis of the Alzheimer's disease peptide Aβ(1-42)
Emelie Wesén, Richard Lundmark, Elin K. Esbjörner.
Submitted

Additional papers not included in this Thesis:

V. Binding of thioflavin-T to amyloid fibrils leads to fluorescence self-quenching and fibril compaction
David J. Lindberg, Anna Wenger, Elin Sundin, Emelie Wesén, Fredrik Westerlund, Elin K. Esbjörner.
Biochemistry, 56(16), (2017), 2170-2174. doi: 10.1021/acs.biochem.7b00035

VI. Lipid membranes catalyse the fibril formation of the amyloid-β (1–42) peptide through lipid-fibril interactions that reinforce secondary pathways
David J. Lindberg, Emelie Wesén, Johan Björkeroth, Sandra Rocha, Elin K. Esbjörner.
BBA Biomembranes, 1859(10), (2017), 1921-1929.
doi: 10.1016/j.bbamem.2017.05.012

[1] All papers are published with my maiden name Emelie Wesén.

CONTRIBUTION REPORT

My contribution to the papers appended in this Thesis is as follows:

I. I conceived the idea together with E.KE, planned the study and performed all the experiments. I analysed the data and wrote the paper together with E.K.E.

II. I conceived the idea together with E.KE, planned the study and performed the cell experiments together with X.Z. I did not partake in the α-syn preparation, AFM or toxicity measurements. I analysed the data and wrote the paper together with D.B. and E.K.E.

III. I conceived the idea together with E.KE, planned the study and performed the experiments. I did not partake in the AFM or ToF-SIMS measurements. I analysed the data and wrote the paper together with E.K.E.

IV. I conceived the idea together with E.KE, planned the study and performed all the experiments. I analysed the data and wrote the paper together with E.K.E.

Preface

This dissertation is submitted for the partial fulfilment of the degree of doctor of philosophy. It is based on the work carried out between June 2014 and February 2020 (with 1 year interruption in 2018 for parental leave) at Chalmers University of Technology, Department of Biology and Biological Engineering, under the supervision of Associate Professor Elin K. Esbjörner. The research was funded by the Swedish Research Council, the Hasselblad foundation, the Wenner-Gren foundation, and the Swedish Research Council funded Linnaeus Centre SUPRA.

Emelie Vilhelmsson Wesén

January 2020

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LIST OF ABBREVIATIONS

α7nChR	acetylcholine receptor α7 nicotinic cholinergic receptor
α-syn	α-synuclein
Aβ	amyloid-β
Aβ(1-40)	amyloid-β 1-40
Aβ(1-42)	amyloid-β 1-42
AD	Alzheimer's disease
apoE	apolipoprotein E
APP	amyloid precursor protein
BACE1	β-site APP-cleaving enzyme 1
β-CTF	β-C terminal fragment
CCP	clathrin coated pit
CCV	clathrin coated vesicle
CIE	clathrin-independent endocytosis
CLIC	clathrin-independent carrier
CLSM	confocal laser scanning microscopy
CME	clathrin-mediated endocytosis
CPP	cell penetrating peptide
CS	chondroitin sulfate
CSPG	chondroitin sulfate proteoglycan
DA	dominant active
DN	dominant negative
ER	endoplasmic reticulum
FEME	fast endophilin-mediated endocytosis
FLIM	fluorescence lifetime imaging microscopy
FRET	fluorescence resonance energy transfer
GAG	glycosaminoglycan
GEEC	GPI-anchored protein-enriched endosomal compartment
GPI	glycosylphosphatidylinositol
HS	heparan sulfate
HSPG	heparan sulfate proteoglycan
HFIP	hexafluoro-2-propanol
LAG3	lymphocyte-activation gene 3
LB	Lewy body
MCI	mild cognitive impairment
MVB	multivesicular body
NMDA	N-methyl-D-aspartic acid
PD	Parkinson's disease
PFF	pre-formed fibrils
PG	proteoglycan
PMT	photomultiplier tube
Trf	transferrin
UV-vis	ultraviolet-visible
VLD	vacuole-like dilation
WT	wild-type

1 Introduction

1 Introduction

The high and continuously increasing number of patients suffering from dementia is an increasingly difficult societal problem and challenge to current health care systems world-wide. An estimated 50 million people live with this syndrome, a number which is projected to increase to over 150 million by 2050 [1] much as a consequence of increased life-expectancy, especially in low-income countries. To date, there is no treatment available that cures or alters disease progression of dementia, one of the major causes of disability, dependency, and suffering among elderly people. The majority of disease cases, 60-70 %, are represented by patients diagnosed with Alzheimer's disease (AD) [1]. The underlying molecular pathology of AD and other dementias, as well that of certain movement disorders such as Parkinson's disease (PD), is neurodegeneration - progressive loss of structure and function of the brain. The most conspicuous pathological hallmark of both AD and PD, as well as many other diseases, is the aggregation of specific proteins into highly ordered, β-sheet rich fibrillar structures known as amyloid fibrils, and the subsequent deposition of these fibrils into characteristic insoluble deposits in afflicted regions of the brain. In AD, these are formed from the amyloid-β (Aβ) peptide and deposited as extracellular plaques, in PD intracellular deposits are formed from the α-synuclein (α-syn) protein.

The formation of extracellular plaque deposits in AD brain has been recognised for over 100 years, when Aloysius Alzheimer first described them [2, 3], and their main proteinaceous component Aβ [4] and its presence in the form of amyloid fibrils [5] was established ~ 30 years ago. Although researchers have intensely attempted to establish the molecular and mechanistic links between Aβ plaque deposition and disease development and progression, a disease-modifying treatment still does not exist [6] and efforts to halt disease progression by decreasing plaque burden has shown questionable clinical efficacy [7, 8], although plaques can be effectively removed [9]. Still, the Aβ peptide indeed plays an important, and seemingly driving, role in AD pathology; this can for example be seen from the high number of AD risk genes directly involved in the production and processing of Aβ [10, 11]. The development and extent of plaque burden does, however, correlate poorly with cognitive decline, and it rather appears to be early aggregated structures, or aggregation-related events, that represent the underlying disease-driving toxic species. In addition, intraneuronal accumulation of Aβ in intravesicular locations has been found to be an early event in disease pathology, appearing before the formation of plaque deposits [12]. This, in combination with endolysosomal abnormalities observed in AD pathology [13] as well as the high number of AD risk genes associated with endolysosomal trafficking [14] has put intracellular Aβ accumulation and trafficking in the spotlight as potential sites for initiation of AD pathology. Further, accumulation in endolysosomal vesicles expose the peptide to aggregation-promoting conditions, and indeed the peptide has been found to aggregate within these locations [15, 16]. To better understand these processes, and their potential link to disease development, it is important to elucidate how Aβ is first internalized into the cell, knowledge that could also be important for the development of future disease-modifying treatments. In addition, there is partial overlap in the pathology of neurodegeneration for different amyloid-related diseases, and by increasing mechanistic

understanding of a certain disease, there is hope for a more profound understanding of neurodegenerative pathology in general.

The aim of my work has been to contribute to the understanding of cellular uptake and accumulation of the amyloidogenic proteins Aβ and α-syn, with a particular focus on Aβ. Such mechanistic understanding is important as a foundation for more profound comprehension of the build-up of intracellular Aβ and how this relates to disease development and progression, with the long-term goal of facilitating the development of future disease-modifying treatments. The Thesis is based on the work presented in four research papers; hereafter denoted as **paper I-IV**. In these, I have studied how Aβ variants (Aβ(1-40) and Aβ(1-42)) influence quantitative aspects of endocytosis of highly monomeric preparations of the peptide (**paper I**), as well as the importance of fibril length for the internalization and toxicity of α-syn (**paper II**). Further, I have explored how the presence of cell surface proteoglycans (PGs) drive the cellular uptake of Aβ, with a focus on the temporal evolution (kinetics) of this relationship (**paper III**). Lastly, I have mapped key protein players and components of different endocytic paths to better understand their contribution to Aβ endocytosis (**paper I** and **IV**). This Thesis begins with an overview of the field in Chapter 2. This includes the concepts of protein folding and misfolding, and the link of the latter to disease. The importance of Aβ and α-syn in disease pathology is described, followed by an overview of endocytosis in the mammalian cell and the internalization of Aβ and α-syn. Brief descriptions of the main methods applied are provided in Chapter 3, followed by a condensation and discussion of the most important results from **paper I-IV** in Chapter 4, and concluding remarks and outlook in Chapter 5.

2 Background

2 Background

In this chapter I will give an overview of the fundamental concepts underlying the work presented in this Thesis. The first section introduces protein biochemistry and the formation of amyloid fibrils by protein misfolding, followed by sections outlining the proteins Aβ and α-syn and their importance in AD and PD, respectively. Lastly, focus is shifted to endocytosis in mammalian cells, outlining mechanisms of endocytosis and the pathobiological motivation for studying endocytosis of amyloidogenic proteins.

2.1 Amyloidogenic proteins

2.1.1 Protein structure and folding

Proteins are the macromolecular units that perform nearly all functions of the cells in our bodies. They are also important cellular building blocks – in fact so highly abundant that they constitute most of the cell dry mass [17]. The structure of proteins is in principle elegantly simple: they form from a sequence of building blocks, amino acids, that are linked together into a linear chain. The astonishing diversity of proteins arises from the combination of these amino acids; there are 20 amino acids found in proteins and considering the median length of 375 amino acids for proteins found in humans [18] there is an enormous complexity in how these units can be arranged. A protein that has acquired its native fold, meaning the arrangement of its amino acids in three-dimensional space to form a functional unit, can exert its function in the cellular machinery. These functions can range from performing enzymatic reactions or acting as antibodies, to cellular transport and signal transmission. Proteins can also convert from, or fail to fold into, their native functional state and instead transition to a misfolded state. This can lead to various pathological conditions, among these are multiple severe neurodegenerative disorders, where proteins are misfolded into highly ordered aggregates.

2.1.1.1 Amino acids and the peptide bond

Proteins, also called polypeptides, are formed by the coupling of amino acids into a polymer via covalent bonds. Proteins are typically > 50 amino acid long polypeptides, whereas shorter structures, < 50 amino acids long, are referred to as peptides. The building blocks of proteins, the amino acids, consist of an amine group and a carboxyl group linked to a central α-carbon (Figure 1). The amine and carboxyl groups participate in the bonding of two adjacent amino acids to form the peptide bond. Linked to the central α-carbon is also a functional group (R) that gives the amino acid specific physicochemical characteristics. There are 20 different amino acids present in proteins, and the characteristics of the R-groups are important in the function and folding of a protein; nonpolar residues tend to be buried in the interior of the protein, and *vice versa* for charged or polar residues. The amino acid, without contribution from its R-group, is a zwitterion at physiological pH as the amine and carboxyl group are protonated and deprotonated, respectively.

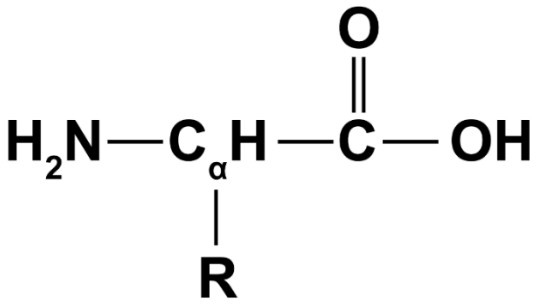

Figure 1. General structure of the 20 amino acids found in proteins.

When amino acids are linked together in a polypeptide chain, a peptide bond is formed by a condensation reaction between the amine group on one amino acid and the carboxyl group on the next. The formed peptide bond has significant double bond character which hinders rotation and renders the bond co-planar. As can be seen in Figure 2, only the two bonds adjacent to the α-carbon are hence able to rotate. The torsion angles of these two are denoted as ψ and ϕ. Because of steric collisions between atoms within each amino acid, most rotations are unavailable. A Ramachandran plot of ψ versus ϕ can be used to depict pairs of rotation angles within a protein [19]. The polypeptide end which has an available amine group is known as the N-terminus, and the other end exposing a carboxyl group is known as the C-terminus.

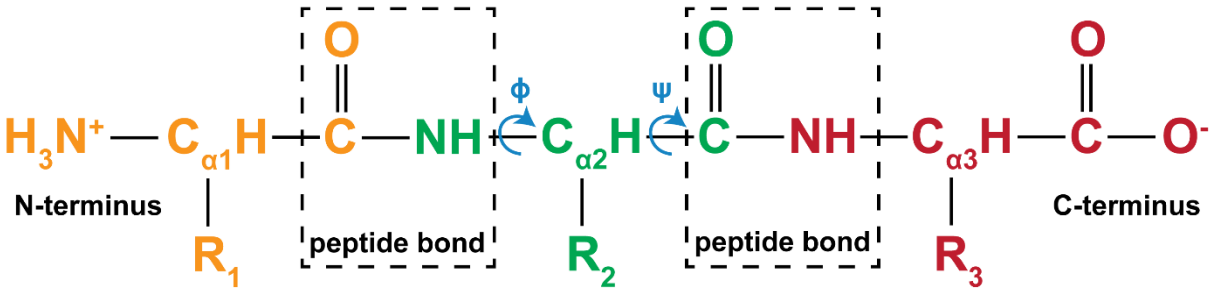

Figure 2. Three amino acid residues (orange, green and red) linked together through peptide bonds.

2.1.1.2 Protein structure

The structure of a protein can be described at different levels. Primary structure is the sequence in which the amino acids are linked together though peptide bonds in the polypeptide chain. The polypeptide can fold up and arrange into three-dimensional structural units that are energetically favourable, and these small structural motifs are what is known as the secondary structure of the protein. Steric constraints, posed by collisions between atoms and the co-planar peptide bond, minimizes the pool of available repetitive structures that the peptide backbone can adopt, and the two most common secondary structures are α-helix and β-sheet (Figure 3). Both are repetitive structures stabilized by hydrogen bonds between N-H and C=O groups in the peptide backbone. The α-helix is a secondary structure where a single polypeptide twists around itself to form a cylindrical structure with a hydrogen bond formed between every forth peptide bond such that the peptide backbone forms a helix that turns one whole revolution every

3.6 amino acids. The β-sheet is also stabilized by hydrogen bonds between N-H and C=O groups of peptide bonds, but this secondary structure is formed by polypeptide chains that run next to each other, either in a parallel or antiparallel manner. Parallel β-sheets typically form when two separate polypeptide chains are aligned, running in the same direction (although they can also form from a single polypeptide chain), whereas antiparallel β-sheet form from a single polypeptide chain that folds back and forth upon itself such that each section of the chain runs in opposite directions to its neighbour. The polypeptides that are the focus of this Thesis, the amyloid-β peptide and the α-synuclein protein, form highly ordered β-sheet rich amyloid fibrils, characterised by a specific cross-β fold, as will be described in more detail in section 2.1.2.2.

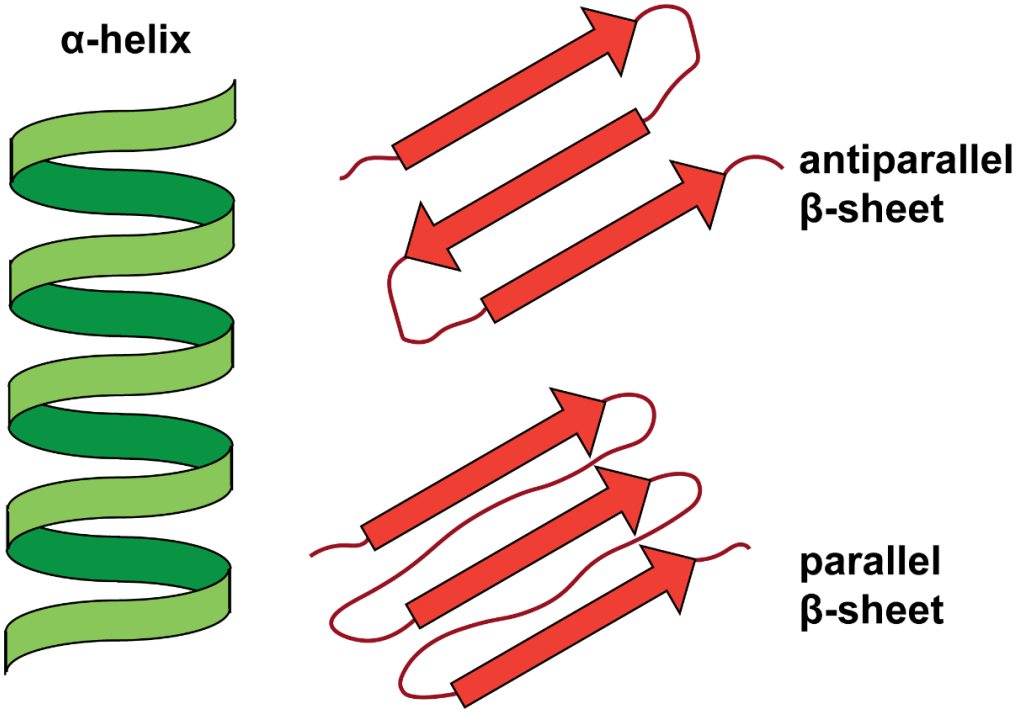

Figure 3. Schematic illustration of an α-helix and two variants of β-sheet; antiparallel and parallel.

The tertiary structure of a polypeptide chain refers to its full fold into a tree-dimensional shape; this can include several folded subdomains of α-helix and β-sheet. Lastly, proteins can consist of several subunits of individual polypeptide chains that arrange together in a three-dimensional shape to form the functional protein; the arrangement of these units is referred to as the quaternary structure of the protein [17].

2.1.1.3 Protein folding

All the information required for a protein to adapt to its functional three-dimensional form is encoded in its sequence of amino acids [20]. The process of these events, transitioning a synthesized polypeptide chain to its native functional form, is called protein folding. The native, final folded structure of a protein is typically the conformation that minimizes its free energy, and proteins thus generally have one single native fold. There are several factors that promote proteins to minimize their free energy and convert to their native form [21]; in addition to adopt

preferred rotation angles to avoid steric hindrance, another is the already mentioned formation of hydrogen bonds, both within and between polypeptide chains. Additional weaker type of interactions that also plays an important role are van der Waals forces; atoms within folded proteins are typically tightly packed which makes these types of interactions an important aspect to take into consideration. Both electrostatic and additional hydrophobic interactions are also important, based on the R-groups of the individual amino acid residues. Hydrophobic residues tend to bury in the interior of the protein, or, oppositely, for membrane spanning proteins, be exposed to the hydrophobic tails of the lipid membrane. This last point, folding to facilitate preferential orientation of polar and hydrophobic residues, has been shown to be particularly important: there is in fact typically not more than a few different conformations a protein can adopt to effectively enable this [22, 23].

A protein could, in theory, adopt a very large number of different conformations, still proteins tend to fold into their native form very quickly, with some proteins observed to fold as fast as on the sub-microsecond time scale [24]. This seemingly opposing characteristics was formulated by Cyrus Levinthal in 1968 [25] and is now known as Leventhal´s paradox: finding the native folded state of a protein by random search would take a very long time, still proteins fold very fast [26]. The energy landscape theory of protein folding can be applied to aid description of this [21, 27], depicted schematically in Figure 4 as a folding funnel. The y-axis represents the free energy of the individual conformation and the width in x relates to the number of possible conformations. There are many open unfolded structures, but as the protein move down the folding funnel the landscape become narrower with only a few, native-like conformations. The protein folds by taking random steps, downhill in energy to reach the native state. In this process it can go via intermediate or partially folded states, sometimes referred to as molten globules, on its path to the correct fold [27]. In the cell, molecular chaperones often assist in protein folding. These helper proteins do not change the final, native conformation of the protein, but rather make the process more reliable by binding to partially folded states and assist them to fold along the most energetically favourable path, thereby also preventing the formation of protein aggregates [17].

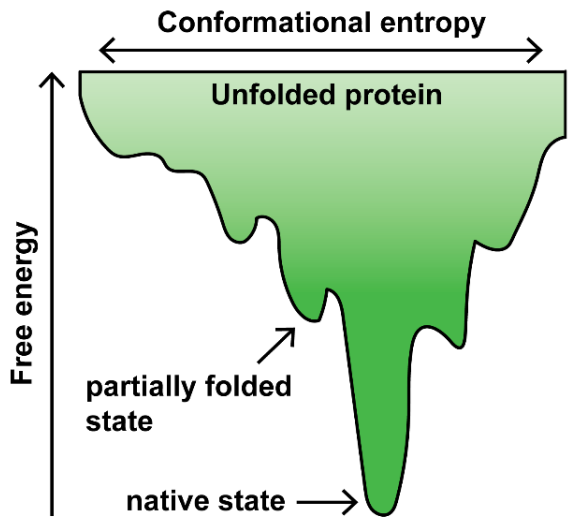

Figure 4. Illustration of the funnel theory of protein folding, depicted as the energy landscape of protein folding.

Successful predictions of folding corresponding to experimentally derived native conformations have been made with relative small proteins [28], and in 2019 AlQuraishi [29] published a promising approach to protein structure prediction based on machine learning using a single neural network that resulted in fast and accurate predictions. Recent advances have also facilitated the use of structure predictions in the design of novel proteins with arbitrary chosen three-dimensional structures [30, 31]. However, although all information required for proteins to adapt their native fold is in principle found in the primary structure, it is still very difficult to predict the three-dimensional native state. Taking the leap from primary sequence to three-dimensional structure is a major challenge in biology, driven by its promise as a tool in understanding cellular functions and disease mechanisms, as well as its potential for enabling structure-driven approaches in drug development.

2.1.2 Protein misfolding and amyloid fibrils

Proteins fold to reach their native, functional state. However, proteins can also, under certain conditions, convert from or fail to form their native state and instead misfold, sometimes leading to the formation of highly ordered fibrillar aggregates [32]. This behaviour is linked to several severe human diseases where a specific protein is misfolded and deposited. This section describes protein misfolding and how it is related to human disease, including the structure and formation of amyloid fibrils, the protein aggregates that are typical in protein misfolding diseases.

2.1.2.1 Protein misfolding and amyloid diseases

Figure 4 depicts a schematic illustration of the protein folding landscape. However, as proteins can also misfold, I will now extend this illustration (Figure 5); at certain conditions proteins can transition into the misfolding and aggregation part of the energy landscape, forming intermolecular contacts, ultimately resulting in the formation of aggregated and highly ordered β-sheet rich species known as amyloid fibrils. The structure of amyloid fibrils will be explained in greater detail in section 2.1.2.2, but it can be noted that they are typically very low in energy, lower than the native fold, and hence very stable. Formation of these species generally proceeds via formation of smaller β-structured aggregates, also known as oligomers or seeds [33]. These can be compared to the intermediate, partially folded, states in the non-aggregating part of the energy landscape. Oligomers can be on- or off-pathway, as designated by whether they proceed to mature amyloid fibrils or not.

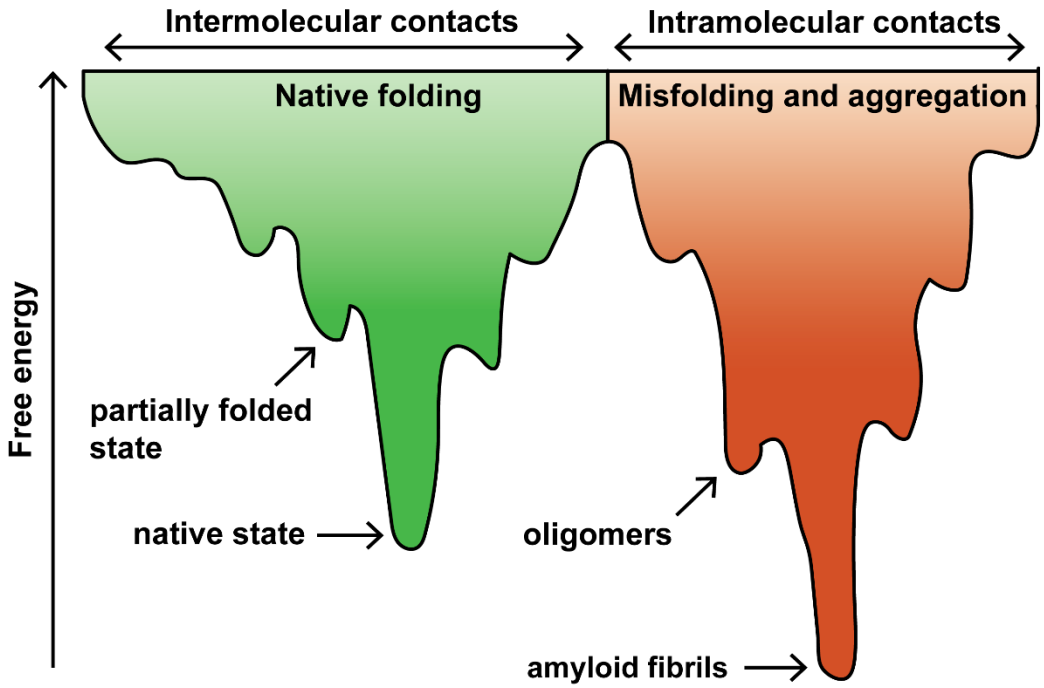

Figure 5. Protein folding landscape including misfolding and aggregation through intramolecular contacts.

There are several severe human diseases associated with the misfolding, aggregation and deposition of proteins, among these are the neurodegenerative diseases Alzheimer's disease (AD) and Parkinson's disease (PD). Each type of misfolding disease is generally associated with the deposition of one or a few proteins that are characteristic for that disease; in AD extracellular depositions of the amyloid-β peptide are formed, and in PD the depositions are found intracellularly and consist of the α-synuclein protein. A total of 37 peptides or proteins are known to be associated with protein misfolding diseases and the formation of amyloid deposits in the intracellular- or extracellular space [33]. In addition, the same protein can be associated with several diseases. Interestingly, the peptides and proteins involved in disease-associated amyloid formation do not have any evident similarities in sequence, structure and function [34]. In fact, 85 years ago it was speculated that fibrillation was a general property accessible for all proteins [35-37]. To effectively form amyloid fibrils, the protein however needs to have amyloidogenic segments [38] that can be exposed as a result of for instance partial unfolding. Another prerequisite is also conformational freedom of this segment so that it can form contacts with additional molecules [36]. Although no apparent sequence or structure similarities are observed in the proteins deposited in amyloid misfolding diseases, they are generally shorter than non-amyloidogenic proteins [34] and approximately half of the proteins known to form amyloid fibrils in their natural biological context are in fact intrinsically disordered [33], meaning that they lack a well-structured three-dimensional fold or have extensive disordered regions [39].

Both amyloid-β (Aβ) and α-synuclein (α-syn), the amyloidogenic proteins at focus in this Thesis, aggregate in the central nervous system and are related to neurodegenerative disease. Protein misfolding diseases can, however, manifest in other tissues as well. In fact, 30 of the proteins involved in protein misfolding diseases form deposits in other tissues; half of these in

specific tissues and the other half in a range of tissues, giving rise to so-called systemic amyloidosis [33]. By understanding more about specific variants of protein misfolding diseases, there is hope for increased understanding of both specific and general disease mechanisms involved in these pathologies.

2.1.2.2 Structure and formation of amyloid fibrils

The protein aggregates that characterize misfolding diseases such as AD and PD are constituted by assemblies called amyloid fibrils. This term was introduced in 1854 by Rudolph Virchow, and as the structures where first thought to be starch-like, he denoted them "amyloid". ~ 30 years later they were shown to be high in nitrogen content and consist of protein rather than carbohydrates [40]. Today amyloid fibrils are defined as an unbranched protein fiber, with a repeating substructure that consists of β-sheets that run perpendicular to the fiber axis [41]. When examined by X-ray diffraction, amyloid fibrils give a very typical diffraction pattern known as cross-β in which two major reflections at ~ 4.7 Å and ~ 10-11 Å are detected [42, 43]. The first of these corresponds to the interstrand spacing and the second to the distance between stacked β-sheets (Figure 6) [41, 43, 44]. These repeating structures align to form so-called protofilaments, typically ~ 2-7 nm in diameter, that each can contain several aligned repeats of β-sheets. The protofilaments in turn twist around each other to form amyloid fibrils, generally 7-13 nm in diameter and built up from a varying number of protofilaments, typically 2-6 [32, 33]. The total length of amyloid fibrils can be several micrometers [45].

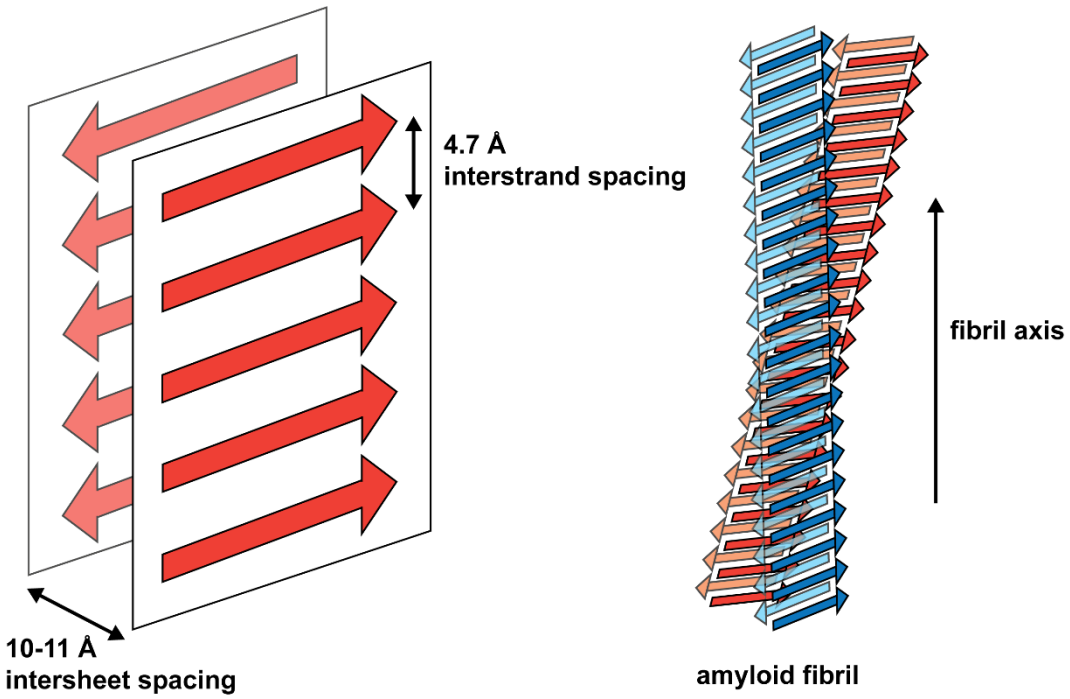

Figure 6. Illustration of amyloid fibril structure. The left image displays typical arrangements of β-structure within an amyloid fibril with distances between individual β-strands and β-sheets, respectively. The right image is an illustration of a possible amyloid fibril arrangement, here depicted as two protofilaments, each consisting of two β-sheets, twisting around each other to form the fibril. It can be noted that the number of protofilaments are variable. The protofilaments can also consist of a variable number of β-sheets.

As an example, the Aβ peptide has, in some of the most recently solved structures, been observed to form protofilaments with cross sections consisting of two monomers, each monomer contributing with four β-sheet regions [46, 47], although other arrangements have also been proposed. The N-terminus of the peptide is observed to be unstructured and is found at the periphery of the protofilament, whereas the C-terminus forms its core [48, 49]. The number of protofilaments forming the amyloid fibril seems to be variable for different Aβ species; the 40 residue variant of Aβ has been observed to form from two protofilaments, whereas the 42 residue variant was observed to form from only one [48]. However, this also appears to be highly variable depending on conditions; amyloid fibrils from the same peptide variant have been shown to display polymorphism for instance with respect to the number of protofilaments forming a fibril or their packing geometry [50]. An interesting example is also the polymorphism observed in a recent publication by Kollmer et al [51], where *in vivo* derived Aβ fibrils where found to be right hand twisted, in contrast to left hand twisted fibrils formed *in vitro*; this shows that fibrils are capable of adapting a larger number of different conformations than previously thought.

To better understand how amyloid fibrils relate to disease, it is of importance to understand how and why these species form. The *in vitro* formation of amyloid fibrils has been extensively studied, and using analytical tools it is nowadays possible to determine the kinetics of amyloid formation in detail [52]. Amyloid formation typically follows sigmoidal kinetics [42]. The process starts with primary nucleation, the formation of so-called nuclei [33]. Nuclei are defined as the smallest structures that can initiate fibril elongation by addition of monomers at a pace which is higher than that of monomer release [53]. Fibrils grow from primary nuclei via monomer addition, but the kinetics of aggregation is also accelerated by new nuclei formed via secondary fibril-dependent events. Secondary nuclei can be formed on the surface of existing fibrils, or alternatively generated by fibril fragmentation, where mechanical cleavage of existing fibrils produces an increased number of available growth ends. The strong influence from secondary pathways on the rate of fibril formation typically leads to the sigmoidal growth kinetics. *In vitro*, the process eventually reaches an equilibrium phase where total amyloid content of the solution is constant [33, 54]. Other early species formed during amyloid formation are metastable intermediates; oligomers consisting of a few monomers and small fibrils with a length of up to ~ 200 nm known as protofibrils [42]. These small species are of great interest as they have been suggested as important toxic assemblies in the pathology of protein misfolding diseases [55]. Insights into the mechanisms and intermediate states of amyloid formation, as well as the interaction of these species with cells, is of great importance to facilitate understanding of disease relevant events and development of potential therapeutic treatments.

2.2 Alzheimer's disease

Alzheimer's disease (AD) is the most prevalent cause of dementia, with an estimated ~ 40 million people affected world-wide [56]. This number is projected to double every 20 years as a result of increasing life-expectancy [57]. The prevalence of AD is strongly related to age; the prevalence is below 1 % in individuals aged 60-64 years [10] but increasing to 10 % at age 65 years and older, and to > 30 % above 85 years [58]. In reality, these numbers are probably underestimated, as pathological changes to the brain can be observed already two decades before the onset of symptoms [56, 59]. Despite these numbers and the large body of research that has been put into finding a disease-modifying treatment for AD, still no curative treatments are available [6] and further research is needed to understand the cellular and molecular mechanisms that underpin the development of AD. The experimental work that this Thesis is built upon concern the AD-related peptide amyloid-β (Aβ) and its interaction with cultured cells, and I will therefore provide a foundation for this work by explaining the pathology of the disease with a focus on Aβ, and with emphasis on intracellular Aβ.

2.2.1 The pathology and treatment of Alzheimer's disease

Over 100 years ago, in 1907, Aloysius Alzheimer described the impaired memory and related symptoms of a 51-year-old woman under his care [2, 3]. When the patient died, Alzheimer examined her brain microscopically and described the presence of the plaque deposits and neurofibrillary tangles (Figure 7) that became, and still are, hallmarks of the disease. The term "Alzheimer's disease" was coined three years later [3]. AD is, in addition to the aforementioned pathological hallmarks, characterized by progressive and gradual decline in cognitive function, resulting from the degeneration of neurons and synapses. The senile plaque deposits and neurofibrillary tangles are found extracellularly in the medial temporal lobe structures and cortical areas of the brain [10].

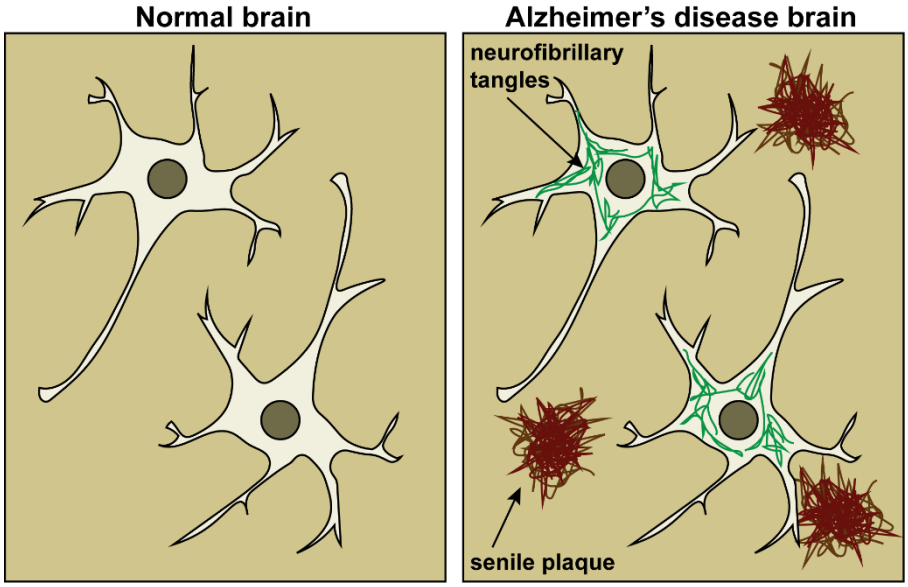

Figure 7. Normal, healthy brain compared to AD diseased brain with the pathological hallmarks of senile plaques and neurofibrillary tangles.

Due to the insolubility of the plaque deposits, it was not until in the 1980s that their protein content was successfully characterized, revealing that the major plaque component was the amyloid-β (Aβ) peptide [4], and that it is present in the plaque deposits in the form of amyloid fibrils [5]. Later, in the 1990s, it was shown that Aβ is produced constitutively and naturally also in non-diseased individuals [60]. Around the same time, the intracellular neurofibrillary tangles, representing the other pathological hallmark of AD, were shown to consist of abnormally hyperphosphorylated tau protein [61].

There are both familial and sporadic forms of AD. Familial AD is an autosomal dominant disorder with onset typically below 65 years of age, accounting for ~ 5 % of disease cases [62]. The plaque and tangle load is typically more severe in familial early-onset AD than in the sporadic form [63]. Familial AD is most commonly caused by mutations in the Aβ precursor protein, amyloid precursor protein (APP) [64], or in presenilin 1 or 2 [65, 66], subunits of γ-secretase that gives the enzyme its catalytic activity to cleave APP to form Aβ. Sporadic forms of AD display later onset than the familial forms, but the age of onset can be lowered by certain risk genes. The apolipoprotein E (apoE) ε4 allele has been shown to account for most of the genetic risk in sporadic AD [67], capable of lowering the age of onset with almost 20 years [10, 68]. In total, over 20 genes have been linked to late-onset AD, and they can be grouped into three general biological classes; cholesterol metabolism, immune response and endosomal trafficking [14]. The latter group affects the membrane trafficking in and out of the early endosome [14], and is thereby directly involved in intravesicular Aβ accumulation. Among trafficking-related genes identified through genome-wide association studies are PICALM [69, 70] and SORL1 [71]. Other potential risk factors of developing the disease are, for example, diabetes, smoking, hypertension, obesity, traumatic brain injury, and lack of cognitive engagement and physical activity [72].

AD is a highly heterogenous disease making both diagnosis and the development of disease-modifying treatments challenging. Aβ accumulation has been shown to start as much as 20 years before onset of symptoms, and substantial neuronal loss is seen already when patients are diagnosed with mild cognitive impairment (MCI) [73], an early but abnormal state of cognitive decline that can be prodromal AD [74]. Clinical diagnosis of AD depends on the history of the patient, neuropsychological testing and assessment of symptoms over time [6]. For a long time, a definite diagnosis of AD could only be made by neuropathology examination post death [75], but recent advances integrating analysis of biomarkers (including volumetric MRI and fluorodeoxyglucose PET) has led to a new set of criteria [6, 76]. To date there is no treatment that prevents, delays or cures the symptoms of AD [6]. In the European Union, only four treatment options are currently approved to treat the cognitive symptoms of AD; among these are three cholinesterase inhibitors and one N-methyl-D-aspartate receptor antagonist [77]. These act to stabilize neurotransmitter disturbances, thereby stabilizing the cognitive function of the patient. These treatments can be effective for up to 2 years, but still they do not change the pathological course of AD [6, 10]. Immunotherapy with antibodies against Aβ, among these aducanumab, are in clinical trials. These have shown to efficiently remove Aβ plaques [78] but still no cognitive improvement was seen in phase III trials [79]. This was however re-evaluated recently, in December 2019, when new data was presented at the 12th Clinical Trials on Alzheimer's Disease conference [80]. Here, it was shown that aducanumab does in fact slow

cognitive decline when supplied at sustained high dose, thus again raising hopes that antibodies could potentially become promising as disease modifying treatments.

2.2.2 The amyloid-β peptide

The Aβ peptide, the main component of AD plaques [4], is formed by proteolytic cleavage of the membrane-bound amyloid precursor protein (APP). There are several isoforms of APP, of which the 696 amino acid residue variant is found in neurons [81]. In the amyloidogenic pathway of Aβ formation, APP is first cleaved by the protease β-secretase (also known as β-site APP-cleaving enzyme 1, BACE1) [82]. This releases the ectodomain and retains the β-C terminal fragment (β-CTF), a 99 amino acid residue long fragment known as C99 [11]. C99 is thereafter further cleaved 38-43 amino acid residues from the newly formed N-terminus by γ-secretase, a protease complex consisting of four components of which presenilin 1 or 2 constitutes the active site [10]. This sequential activity of β- and γ-secretases releases the ~ 4 kDa, intrinsically disordered [83], Aβ peptide (Figure 8). There is also a non-amyloidogenic pathway of APP processing through which most of the protein is processed, at least under normal conditions. This path is initiated by α-secretase cleavage 83 amino acid residues from the C-terminus, within the Aβ region, thereby hindering the formation of Aβ [11].

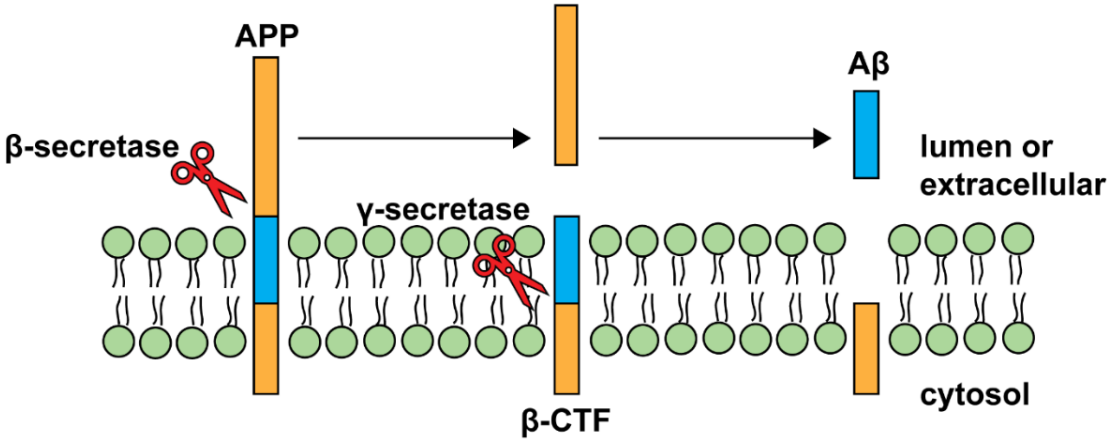

Figure 8. APP processing by β- and γ-secretases resulting in the formation of the Aβ peptide.

Depending on the exact cleavage position of γ-secretase, different Aβ isoforms can be formed. The majority of cleavage events typically results in the formation of the 40 amino acid residue variant Aβ(1–40) (80–90%) and thereafter the 42 residue variant Aβ(1–42) (5–10%) [84]. The more aggregation prone [85] Aβ(1-42) isoform is neurotoxic [86], predominant in extracellular plaques [87] and selectively accumulated in intraneuronal locations [12].

In 1992, Hardy and Higgins proposed the amyloid cascade hypothesis; that the deposition of Aβ into plaques is the causative event in AD pathology, and that formation of neurofibrillary tangles, cell loss and dementia follows as a direct result [88]. This hypothesis has been revised over the years [62], much due to the lack of correlation between extracellular plaque load and degree of dementia [89]. It is now commonly thought that Aβ exerts its pathological role earlier,

before the deposition into insoluble plaques [84, 90]. Intracellular accumulation of Aβ could contribute to disease progression [12, 91], a topic which will be further discussed in the next section. Independent of the exact mechanism of Aβ involvement in disease development and progression, the peptide indeed plays a very central pathological role in AD. In support of this are the many Aβ-related mutations that are linked to familial AD; both APP-mutations within or immediately flaking in the Aβ-regions and mutations in γ-secretase leading to increased formation of the more aggregation-prone Aβ(1-42) [10, 11, 56]. In addition, increased levels of APP through locus duplication leads to the development of early onset AD [92], and triplication of chromosome 21, on which APP is located, in Down syndrome results in intracellular accumulation of Aβ and plaque pathology at young age [93].

2.2.3 Intracellular Aβ

The presence of Aβ in extracellular plaques has been known since the 1980s [4], and not long after, in 1989, came the first report on intraneuronal Aβ in both healthy and AD diseased human brains [94]. This first study was performed with an antibody against the 17-24 amino acid residues of Aβ, hence it also reacts to APP and β-CTF. However, in the 1990s, the development of antibodies targeting the C-terminal end of Aβ made it possible to detect Aβ in plaques without the risk of cross-reactivity with APP or β-CTF [95]. Using these antibodies, it was shown that Aβ indeed is present intracellularly in cultured human neurons [96]. Moreover, it has been shown that preferably the more aggregation prone and disease-relevant Aβ(1-42) accumulates in intraneuronal locations and that this accumulation appears to precede plaque formation [12]. This temporal relation, with intracellular accumulation appearing first and plaque deposition secondary, has also been observed in Downs syndrome [93, 97], with the intracellular levels decreasing as plaques build up [97]. Intraneuronal Aβ has historically not been given as much attention as the extracellular Aβ deposition. Interest has, however, increased lately, much due to the findings of intracellular trafficking-related risk genes in AD [14], mentioned earlier. Still it remains unknown exactly how intraneuronal build-up of Aβ is related to AD development. One plausible mechanism is that Aβ assembly begins intracellularly, and that aggregates later on end up in extracellular plaques following neuritic degeneration [98]. This could explain the lack of correlation between plaque load and degree of dementia [89] as well as the presence in plaques of proteins that are otherwise normally found intracellularly [99, 100].

To understand the origin of intracellularly accumulated Aβ, it is important to consider the sites where the proteolytic cleavage events leading to its production occurs (Figure 9). Intracellular accumulation of Aβ could occur both from Aβ being produced intracellularly, and from secreted peptide being re-internalized from the extracellular space [11]. Production and release of Aβ involves the endocytic pathway [101], and APP and the β-secretase BACE1 are both present at the plasma membrane as well as in early endosomes, where they have also been shown to interact [102]. Interestingly, they appear to be transported to early endosomal compartments through different mechanisms [103], suggesting an endocytic regulation of Aβ production. Due to the acidic nature of endosomes and BACE1 having optimal activity at acidic pH, early endosomes are a likely site where BACE1 act to cleave APP [11]. The γ-secretase subunit presenilin-2 has been shown to be directed to late endosomes and lysosomes, thereby producing an intracellular pool of Aβ through cleavage of β-CTF [104].

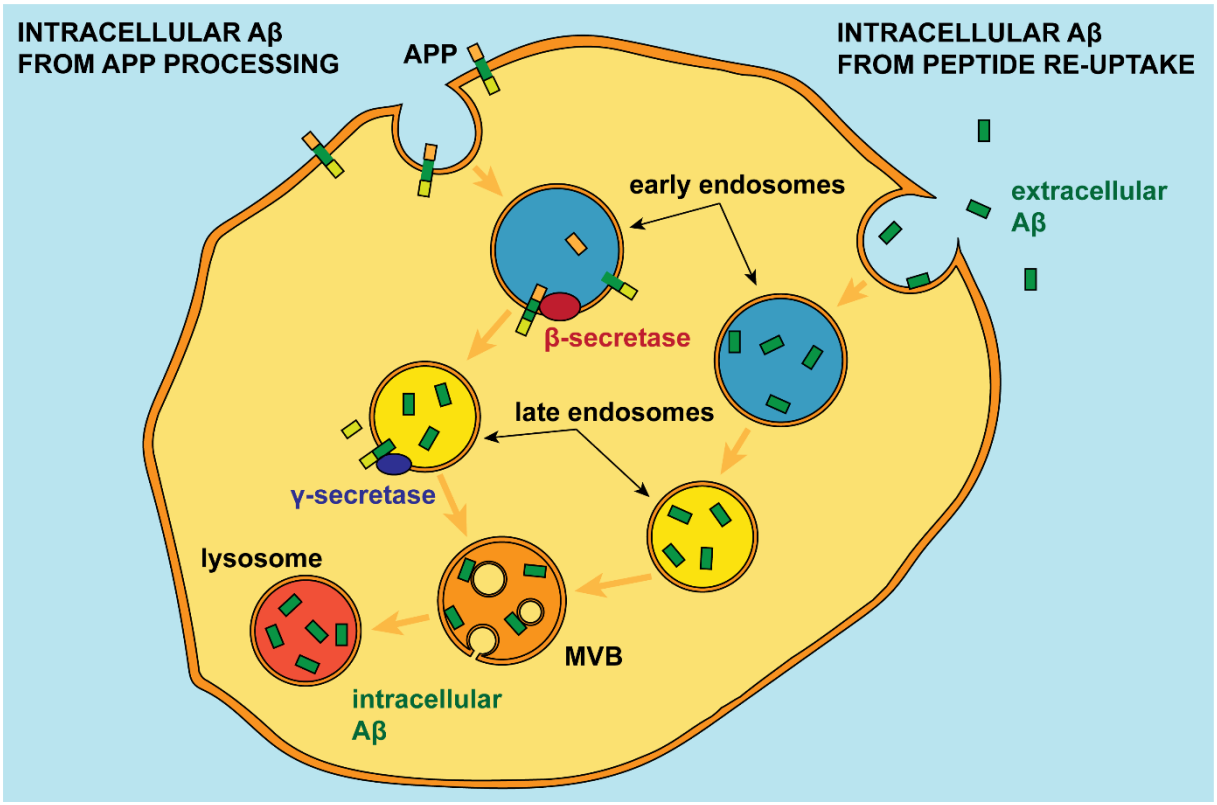

Figure 9. Origins of intracellular Aβ. Aβ can accumulate in vesicles of the endolysosomal system both as a direct result of APP processing, and from re-uptake of extracellular Aβ.

The above findings of Aβ generation in the endolysosomal system agree with the observed intracellular accumulation of Aβ in multivesicular bodies [105, 106], a type of late endosome. In addition to Aβ generation in the endolysosomal system, there is also evidence for that the peptide is produced and secreted though the secretory pathway [107]. There is hence both an intra- and an extracellular Aβ pool, and it has been indicated that a dynamic equilibrium exists between the two [108]. Endosome abnormalities are among the earliest neuropathological features observed in AD [13], and this, in combination with the observation that Aβ can be re-internalised into cells both *in vitro* and *in vivo*, points to involvement of the endolysosomal system in disease development and progression.

2.3 Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disease [109]. The disease holds its name from James Parkinson who described the motor symptoms of the disease in 1817 [110]; tremor (shaking) at rest, bradykinesia (slowness of carrying out movements) and, sometimes, akinesia (inability to move muscles voluntarily) [111]. It is now known that it is the loss of substantia nigra dopaminergic neurons that is responsible for these core motor symptoms [112], and this loss is described as one of the pathological hallmarks of the disease. PD is, however, not only a disabling neurodegenerative disease with motor symptoms; cognitive decline is also an aspect of the disease [109], and at least 75 % of patients surviving > 10 years with the disease develop dementia [113]. The second pathological hallmark of PD is the presence of cytoplasmic inclusions called Lewy bodies (LBs) [111] (Figure 10). These inclusions are present not only in PD, but also in other disorders typically grouped together as Lewy body diseases [114]. The diagnosis of PD is based on a combination of motor features and associated symptoms, and the response to levodopa [115]; a drug that is converted to dopamine in the brain [116]. This drug is currently the most effective among available medications to treat the motor symptoms of PD, but there are still no available disease modifying treatments to alter the underlying progression of neurodegeneration [117].

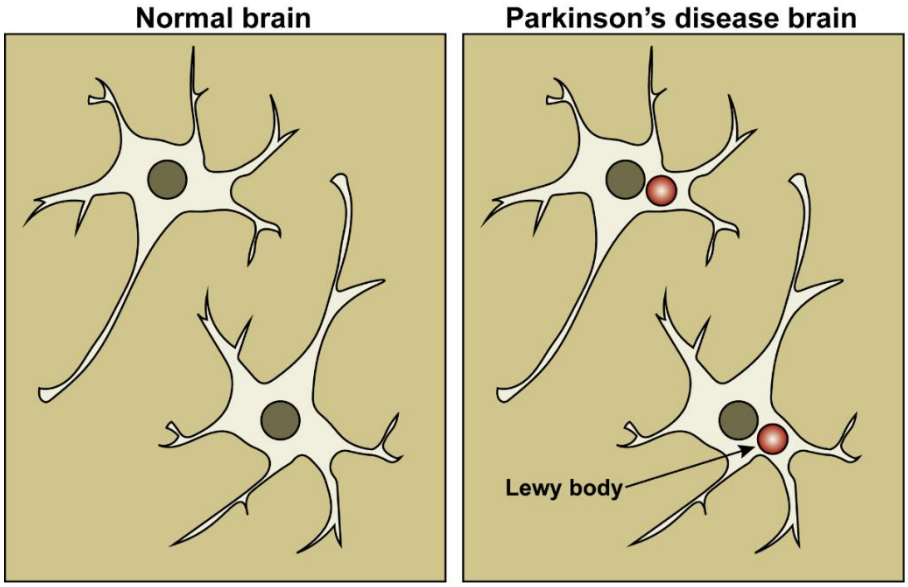

Figure 10. Normal, healthy brain compared to PD diseased brain with intracellular accumulation of α-syn in Lewy bodies.

In the end of the 1990s, the main component of LBs was found to be the presynaptic amyloidogenic protein α-synuclein (α-syn) [118]; a 14 kDa protein consisting of 140 amino acid residues. The normal function of α-syn has not been established, but the protein has been suggested to have a role in neurotransmitter release, synaptic function and plasticity [114]. α-syn is considered an intrinsically disordered protein, as the monomer lacks three-dimensional structure in aqueous solution [119], thereby complicating the search for its native function(s) which may be variable [120]. The protein has an amphipathic N-terminus which is prone to

α-helix formation, thereby facilitating interaction between α-syn and membranes [121], and a disordered and acidic C-terminus. Between these regions, at amino acid residues 65-90, is a highly hydrophobic region that is thought to drive aggregation of the protein [114, 122].

PD, like AD, can be both sporadic and familial, with the latter corresponding to 10-15 % of disease cases, which are also typically early onset [123]. The majority of mutations leading to the familial form of the disease are found in the α-syn gene (SNCA); these can however both result in increased or, oppositely, decreased aggregation rate of the protein [124]. Duplication and triplication of the SNCA gene has been shown to result in early onset PD, with a direct relation between gene dosage and disease progression [125], clarifying the important role of α-syn in development and progression of PD. In addition to being an important component of Lewy bodies, α-syn can also be released from, and re-internalised into, cells [126], thereby propagating aggregates from neuron to neuron via a seeding mechanism [126-128]. To understand these processes better, and facilitate future development of disease-modifying treatments, it is important to understand the mechanisms and pathways that leads to cellular exit and entry of the α-syn protein.

2.4 Endocytosis

This Thesis focuses on the cellular uptake of amyloidogenic proteins in cultured mammalian cells, and in this section I will therefore provide a brief description of the mammalian cell and its central compartments and functions, with a focus on endosomes and cellular uptake via endocytosis. Endocytosis of Aβ and α-syn will be further elaborated, as well as endosomal accumulation of these amyloidogenic proteins as an initiation point for protein aggregation.

2.4.1 The mammalian cell

The mammalian cell (Figure 11) is surrounded by a phospholipid bilayer, the plasma membrane, that defines its boundaries, distinguishing what is outside (extracellular) from what is inside of the cell (intracellular). The plasma membrane and its components, among these receptor proteins and sugars, are important for how the cell interacts with other cells as well as components in the extracellular space. The intracellular part of the cell consists of the cell nuclei containing its DNA, and the cytoplasm surrounding it. The cytoplasm, in turn, consists of organelles and the protein-containing fluid that surrounds them, known as the cytosol. The cytoplasmic organelles are surrounded by phospholipid membranes. DNA is transcribed to RNA in the nucleus, where after it is transported to the endoplasmic reticulum (ER) or directly to the cytosol, where it can be translated to protein. The ER is also, among other things, important in lipid synthesis. The part of the ER containing ribosomes, the factories of protein synthesis, is known as the rough ER, and the part of ER that is absent of ribosomes is known as the smooth ER. The Golgi apparatus is a site where proteins can be further modified, as well as packaged into vesicles for transport, both within the cell and for secretion. To provide the cell with the energy that it needs to perform its functions and respond to cues, the cell has mitochondria. The backbone of the cell is the cytoskeleton; it gives the cell its shape and mechanical resistance. As a consequence of the dynamic nature of the cytoskeleton it can deform cellular membranes and allow the cell to migrate; the cytoskeleton is also very important in endocytosis, the collective name for a series of processes in which extracellular components are captured by the plasma membrane in pockets or protrusions that pinch off from the membrane to form vesicles called endosomes. These endosomal vesicles are then transported, or trafficked, though the endosomal network of the cell. They are in some cases transported to lysosomes, which are the major degradation units of the cell [17].

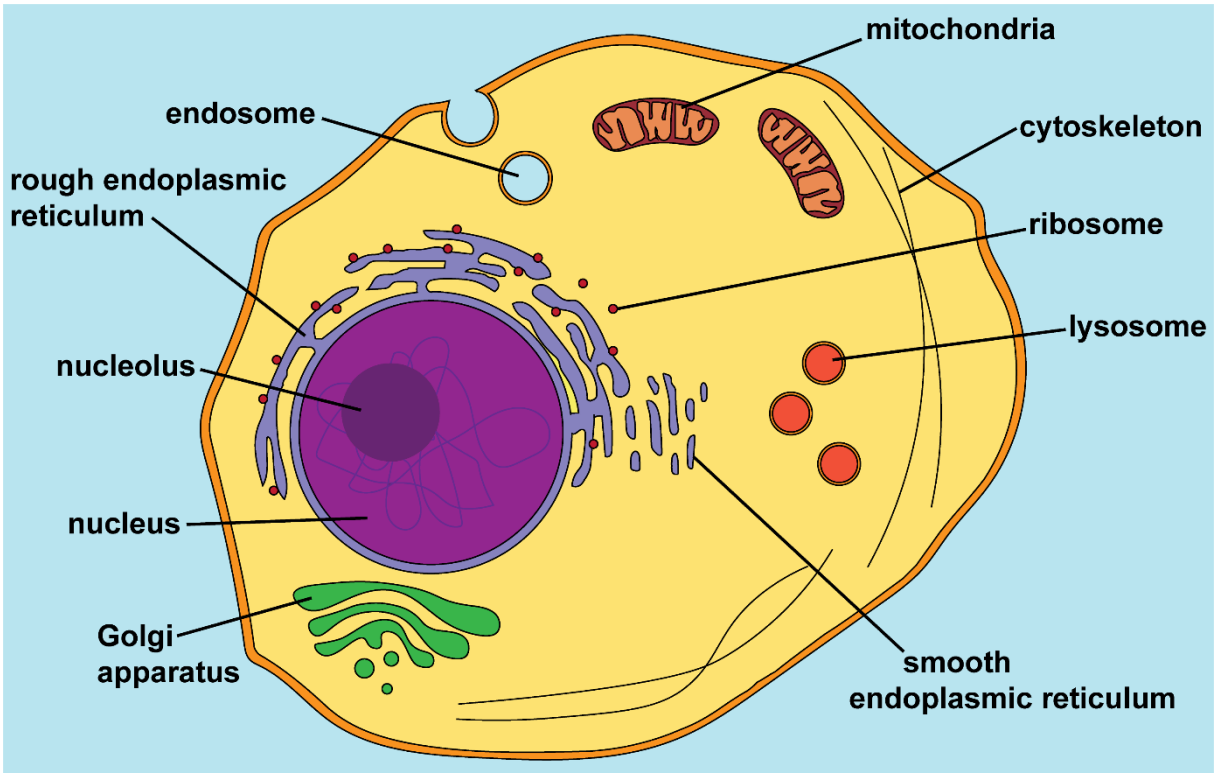

Figure 11. Schematic illustration of a mammalian cell and its organelles.

2.4.2 Endocytic pathways

Endocytosis is the event by which the plasma membrane invaginates and pinches off to form vesicles called endosomes, where the previously external membrane now is an internal membrane [129]. In that sense it can be seen as a direct opposite of exocytosis, during which intracellular vesicles fuse with the plasma membrane, releasing their content to the extracellular milieu [130]. Endocytosis is a process during which the cells internalize extracellular solutes and structures, either as a consequence of them interacting with the plasma membrane components or receptors, or as an indirect event of them being present in the fluid that is internalised. It is also a way for the cell to regulate its plasma membrane composition, the presence of signalling molecules on its surface [131] and membrane tension [132].

Endocytosis can occur via a range of different mechanisms (Figure 12), or endocytic paths. However, once material has been endocytosed, these vesicles merge into a common early, also called sorting, type of endosome. These early endosomes tubulate as a way to facilitate their cargo sorting. The fate of the cargo can thereafter take several different routes; it can be reverted back to the plasma membrane via recycling endosomes, sent to the trans-Golgi network via retrograde transport [133], or traffic through the endolysosomal system via late endosomes/multivesicular bodies (MVBs) to the lysosomes. As vesicles mature along the endolysosomal path they increase in acidification, which in turn partially determines sorting along this path [134].

A first division of endocytic mechanisms can be made based on the involvement of clathrin, i.e. clathrin-mediated endocytosis (CME), and clathrin-independent endocytosis (CIE). CME is the most studied endocytic mechanism, it has well-known ligands, such as transferrin (Trf)

[135], and occurs by a sequence of events that are understood in significant detail. During CME, clathrin forms a coat, or lattice, around invaginating structures of the membrane, known as clathrin coated pits (CCPs). These structures later pinch off from the membrane to form clathrin coated vesicles (CCVs) with the help of dynamin in the scission process [136]. The vesicles formed are uniform in size, ranging between 150-200 nm [137].

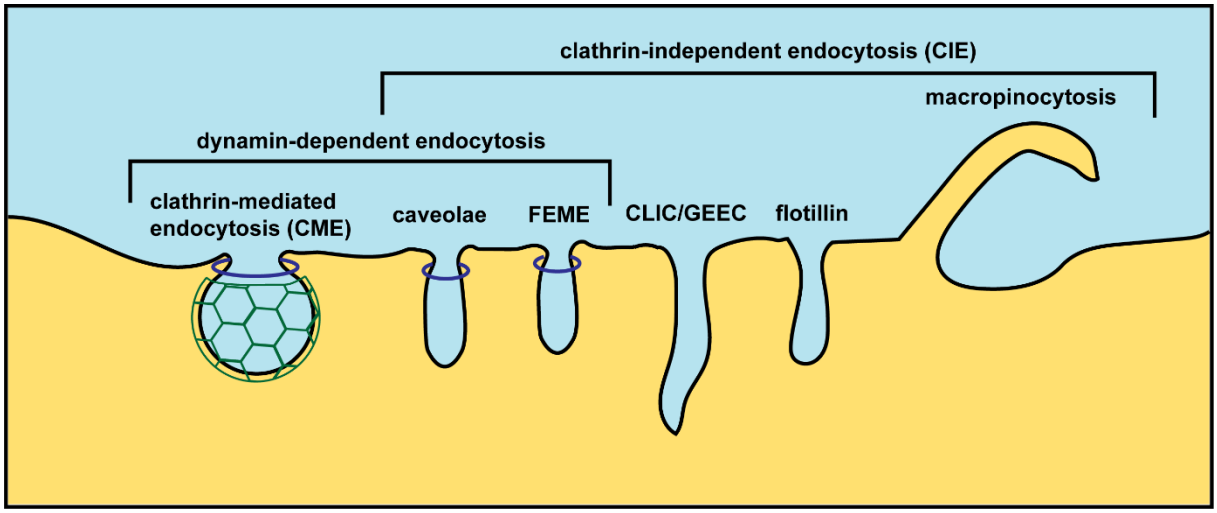

Figure 12. Illustration of different endocytic uptake mechanisms that cells use to internalise molecules.

Dynamin is not only involved in CME, but also in other endocytic mechanisms [129]. CIE can hence be divided into dynamin-dependent and -independent paths. Here it should be noted that CIE is a highly dynamic and developing research field and that CIE uptake display a great deal of complexity. It is for example still not known how many discrete endocytic paths a cell actually has, a question which is also complicated by the fact that the same endocytic regulators can contribute to more than one process [138]. Uptake via caveolae and fast endophilin-mediated endocytosis (FEME) are two dynamin-dependent endocytic paths. Caveolae are small flask-shaped plasma membrane invaginations about 80 nm in diameter [138]. They are enriched in glycosphingolipids and cholesterol, and caveolin is a characterizing protein of these microdomains [139]. There has been a long debate regarding the actual involvement of caveolae in endocytosis [138], and they are now generally considered to be relatively stable structures that are not involved in constitutive endocytosis [139]. Rather, their main function seems to be mechanoprotective; flattening of the invaginations will protect the cell from membrane stress [140]. FEME occurs via tubulovesicular membrane intrusions, prominent at the leading edges of cells and activated upon ligand binding. Rho GTPases are molecular switches that regulate many cellular processes, among these the assembly and organization of the actin cytoskeleton, thereby making them important also in endocytosis [141]. The best characterized Rho GTPases are Cdc42, Rac1 and RhoA [142]. These are not only involved in various endocytic paths [143], but have also been implicated to play a role in AD pathogenesis [144]. The FEME pathway is inhibited by inhibition of Rac1 and RhoA, but activated by Cdc42 inhibition [145] (Figure 13).

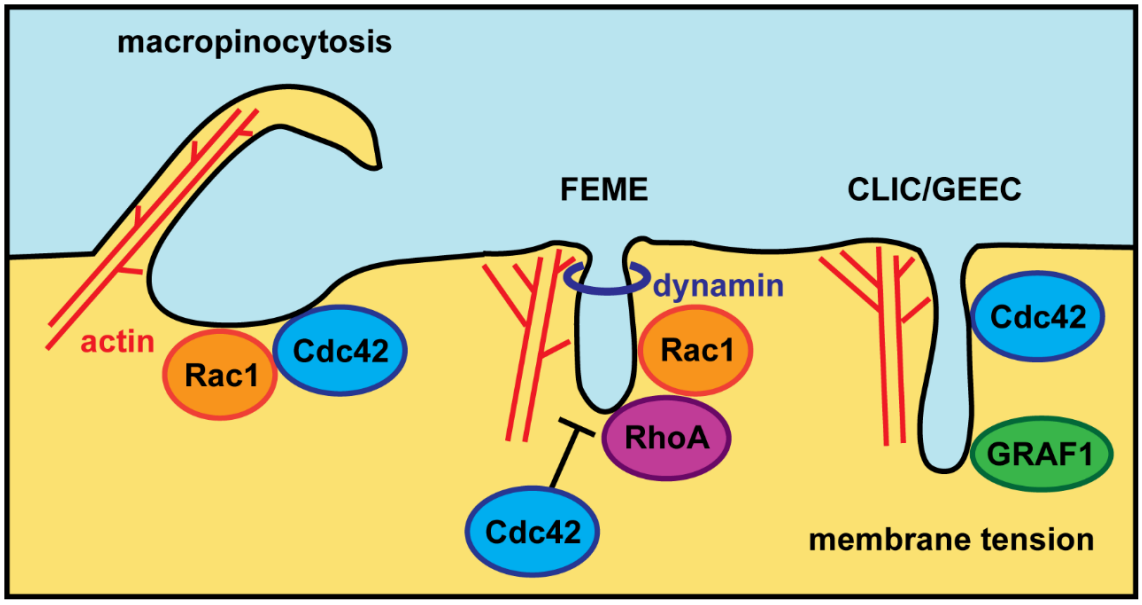

Figure 13. Uptake via the CIE-mechanisms macropinocytosis, FEME and CLIC/GEEC, highlighting involvement of the Rho GTPases Cdc42, Rac1 and RhoA, as well as that of GRAF1 and sensitivity to membrane tension.

Dynamin-independent CIE paths include uptake via flotillin (although in some cases described as dynamin-dependent [146]), CLIC/GEEC (clathrin-independent carrier/glycosyl-phosphatidylinositol (GPI)-anchored protein-enriched endosomal compartment) and macropinocytosis [137, 138]. Flotillin is found in cholesterol-enriched microdomains and has been implicated in endocytosis. It has been proposed that flotillin does not mediate endocytic vesicle formation per se, but rather participate in pre-endocytic clustering of cargo [138]; therefore the term flotillin-assisted (rather than "dependent") endocytosis has been suggested [146]. CLIC/GEEC is another seemingly dynamin-independent path (although, dynamin has been reported to play some role in the process, at least in some cases [147]) which is dependent on Cdc42 but not on Rac1 [148, 149], and that has been found to, at least in part, be regulated by the GTPase-activating protein GRAF1 [147]. Uptake via the CLIC/GEEC pathway is rapidly and specifically upregulated by reductions in membrane tension [132] (Figure 13). Macropinocytosis is a process that is well distinguished from other endocytic paths in that it leads to the formation of large vesicles, varying in size from 0.2 to 10 µm. These vesicles, macropinosomes, form spontaneously or as a response to stimulation, for example of growth factor receptors. Macropinosomes form from cell surface ruffles, which are sheet-like extensions of the plasma membrane, and this process is hence highly dependent on the actin cytoskeleton [150], as well as on Rac1 and Cdc42 [151] (Figure 13). Although macropinocytosis is commonly described as a dynamin-independent uptake path, the formation of circular dorsal ruffles, that appear to be involved in certain types of uptake via macropinocytosis, is dependent on dynamin [138, 152], demonstrating the complexity of CIE and its classification.

2.4.2.1 Cellular uptake of Aβ peptides

Depending on which endocytic path(s) that are used for uptake of a molecule, it may be subjected to different intravesicular settings related to the physical attributes of the specific vesicle, its maturation and intracellular fate. To better understand the build-up of intracellular Aβ and its implications in disease, it is hence important to study its uptake (or, *in vivo*, re-uptake) paths. In this Thesis, I have focused on soluble, mainly monomeric, Aβ preparations; a choice which was based on the are fact that endolysosomal vesicles are sites where Aβ is prone to aggregate (as will be further discussed in section 2.4.4), a process that I aimed to shed light on, and indeed monitored in **paper III**.

There are several neurotransmitter receptors that have been suggested in the uptake of soluble Aβ, among these are the glutamate receptor N-methyl-D-aspartic acid (NMDA) [153] and the acetylcholine receptor α7 nicotinic cholinergic receptor (α7nChR) [154], of which the latter has been of particular interest due to the degeneration of cholinergic neurons in AD [155]. In addition, apolipoproteins, especially apoE [156] and its major receptor low-density lipoprotein receptor-related protein 1 (LRP1) [157], have been suggested to mediate uptake of Aβ. Interestingly, all of these suggested receptors are involved in CME [158]. In studies of the endocytic uptake paths of soluble Aβ both clathrin-dependent [157] and -independent [159, 160] uptake has, however, been observed. Clathrin-independent uptake has been suggested to occur both via macropinocytosis [159], and dynamin-dependent uptake [160], as well as via a non-endocytic path of direct membrane penetration [160]. These are clearly contradicting results, complicated further by the fact that the Aβ peptide source and preparation protocol has differed, making it difficult to assess the aggregation state of the starting material in each study. There is hence a need for further clarifications, and for studies with well characterized Aβ preparations. Also, it cannot be excluded that multiple uptake paths are in fact involved in internalisation of the peptide; whereupon the identification of their relative contributions under different conditions becomes important. An interesting recent report investigated this by studying the chirality dependence of uptake [161]; Aβ uptake in that case appeared to be predominantly receptor-mediated, but seemed to also occur via non stereoselective (~ 20 % of internalised peptide) processes, such as macropinocytosis [161]. There is an apparently greater consensus in the field regarding the uptake of oligomeric forms of Aβ, which are often observed to be dependent on dynamin and RhoA, but yet again independent of clathrin [162-164].

There are, hence, a wide range of receptors and other proteins have been reported to bind to soluble and unaggregated Aβ and facilitate uptake [11, 158]. Based on the aggregating nature of Aβ, and its observed local aggregation at the membrane prior to endocytosis [165], it is not surprising that several endocytic paths appear to be of importance for its internalization, which can also be different in different cell types [158].

2.4.2.2 Cellular uptake of α-synuclein

Cellular internalization of α-syn is thought to play a role in the prion-like spread of PD pathology across the brain [166]. The LAG3 (lymphocyte-activation gene 3) receptor has been shown to bind to α-syn pre-formed fibrils (PFFs, commonly used in the field and fragmented by sonication prior to incubation, yielding fibril fragments of typically < 50 nm average length [167]) and thereby initiate endocytosis, transmission, and toxicity [168]. Compared to Aβ, the published literature on the cellular uptake of α-syn is much more uniform; the consensus is that

α-syn fibrils can enter cells via an endocytic path which is dependent on dynamin [169]. This can be seen from the large body of research that has been performed in various cell types using expression of dominant-negative forms of dynamin and the pharmacological inhibitor dynasore [170-173]. In addition to this, fibril uptake via macropinocytosis [174] and through the interaction with cell surface heparan sulfate proteoglycans [174, 175] has also been demonstrated; the latter will be further discussed in section 2.4.3. Interestingly, the uptake of monomeric α-syn has been suggested to occur via direct translocation though the plasma membrane, rather than via an endocytic mechanism [170].

In this Thesis, the uptake of fragmented fibrillar α-syn of varying lengths were studied in terms of cellular uptake (Figure 14, **paper II**), thereby extending a previous study on the correlation of α-syn toxicity with extent of fibril fragmentation [176]. In relation to these fragmented fibrils, another interesting study on the cellular uptake of α-syn fibril fragments can be mentioned [177]; in this study the capacity of α-syn fragmented fibrils to seed fibrillation of unaggregated cytoplasmic α-syn was assessed in cultured cells, as well as after injection into mouse brain. Fibrils < 50 nm in length were shown to be the most potent species, and it is hence relevant to further characterize the endocytic uptake of small α-syn fibrils as a potential step in disease progression and spreading across the brain.

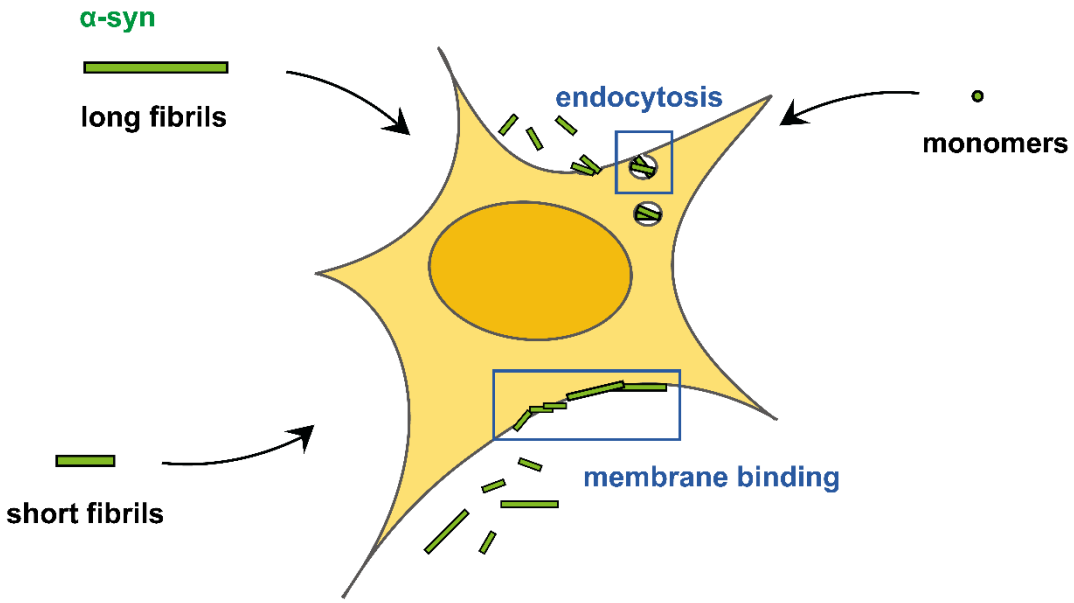

*Figure 14. Schematic illustration of uptake and membrane-binding of monomeric α-syn as well as full length and fragmented α-syn fibrils, as studied in **paper II**.*

2.4.3 Cell surface proteoglycans in uptake and protein aggregation

Glycosaminoglycans (GAGs) are unbranched polysaccharide chains that are built up from repeating disaccharide units. One of the sugars in the disaccharides is an amino sugar, which is often sulfated, and the other sugar is usually an uronic acid; this combination of sulfated and carboxylated sugar units renders GAGs highly negatively charged and hence also hydrophilic [17]. Sulfated GAGs gained interest in the AD research field ~ 30 years ago, when they were

first found to be deposited into extracellular Aβ plaques [178]. GAGs can be of various types depending on the types of sugars and their linkages, as well as the number and location of sulfated groups [17]. It has been shown that the GAGs heparan sulfate (HS) [179] and chondroitin sulfate (CS) [180] are co-deposited with Aβ in plaques.

Proteoglycans (PGs) are assemblies of a core protein with covalently attached GAGs. PGs often have a carbohydrate content of as much as 95 weight %, most of which is in the form of long and unbranched GAG chains. Due to their co-assembly with Aβ, the PGs HSPG and CSPG are of particular interest in relation to AD. PGs can have a range of different activities in the cellular context and many PGs are secreted as components of the extracellular matrix where they, for instance, can be important in signalling between cells. PGs can also be components of the cell membrane, either by their core protein being inserted across the membrane or by it being attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor. These cell membrane-residing PGs can act as co-receptors that collaborate with conventional protein receptors [17], or act as a receptors by themselves [181]. Being components and (co-)receptors of the plasma membrane, PGs are important for cellular uptake via endocytosis. PGs have been proposed to be involved in uptake via clathrin- and caveolin-independent, but flotillin- and dynamin-dependent endocytosis [182], as well as via macropinocytosis [174, 183-185], the latter of which can be triggered by the binding of the amyloidogenic protein tau to HSPGs [174, 184] as well as by PG-clustering on the cell surface induced by binding of cell penetrating peptides (CPPs) [185].

Cell surface PGs are also of interest in relation to AD due to their ability to enhance Aβ amyloid formation [186], more specifically, both HS and CS GAGs were found to catalyse fibrillation [187]. HSPGs and CSPGs have also been observed to be important in Aβ uptake and toxicity. Cells deficient in these cell surface components have been shown to have a decreased Aβ uptake rate [188-190], and, in addition, are less sensitive to Aβ toxicity [188], indicating an intracellular basis of toxicity. In this Thesis I have built on these findings by studying the temporal evolution of PG-dependency in Aβ endocytosis (**paper III,** Figure 15**)**. Also, fragmented amyloid fibrils of α-syn, but not oligomers, have been shown to be dependent on HSPGs for their internalization into cultured cells [175]. In this Thesis, I extend these findings by reporting on the involvement of HSPGs in the endocytosis of fragmented α-syn fibrils of average length 110 nm (**paper II**).

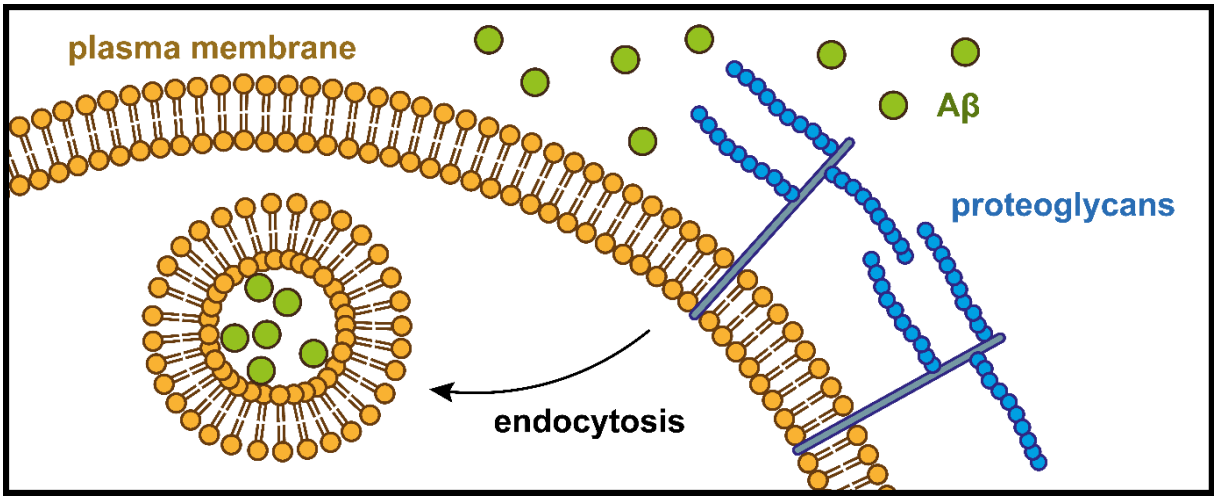

Figure 15. Cell surface PGs are important in Aβ endocytosis, and the temporal evolution of this relation is studied in ***paper III****.*

2.4.4 Endolysosomal vesicles and amyloid formation

Endolysosomal vesicles are not only important sites in Aβ cleavage from APP (see section 2.2.3), they are also sites of Aβ accumulation due to re-internalization from the extracellular space. As amyloid formation is highly concentration-dependent [85], the concentrating nature of endolysosomal accumulation renders these organelles particularly aggregation-promoting. Another important aspect in endocytosis of amyloid-forming polypeptides is their close contacts with the phospholipid membrane surrounding the endosomal content; we and others have shown that lipid membranes catalyse the formation of Aβ amyloid fibrils [191, 192]. In addition to peptide concentration and the presence of a lipid membrane, the third factor further increasing the aggregation propensity of amyloidogenic proteins after endocytic uptake is the acidifying nature of the endolysosomal trafficking system (Figure 16); material being transported through the endolysosomal degradative pathway towards the lysosome will be subjected to a sequentially decreasing pH, from physiological pH at the endocytic event eventually reaching pH ~ 4.7 in the lysosome [134]. This is important, as pH is highly influential on the aggregation propensity of Aβ [193]. The presence of lysosomal proteases could, on the other hand, act to prevent fibrillation or degrade already formed Aβ fibrils [194].

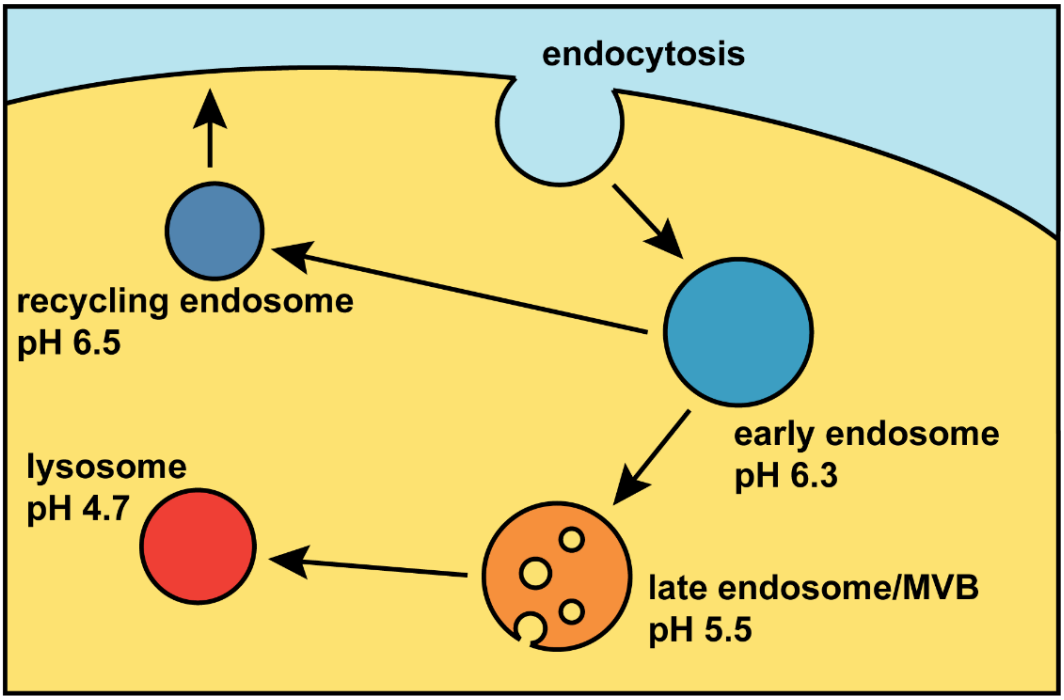

Figure 16. Decreasing pH along the endolysosomal trafficking pathway.

Indeed, in work by Hu et al [15], Aβ has been observed to be internalized at very low concentrations, in the nM range, and concentrated by a factor ~ 100 in endolysosomal vesicles. Further, when applied at µM concentrations, the peptide was observed to have formed amyloid fibrils within endolysosomal vesicles. In previous work within our research group, this amyloid-forming behaviour was followed *in situ* by observations of aggregation in live cells using fluorescence lifetime imaging microscopy (FLIM) [16].

In order to better understand the intravesicular aggregation of amyloidogenic proteins and peptides, such as Aβ, and eventually how this potentially relates to disease development and progression, it is important to deepen understanding of the preceding step; how these species are internalised and transported intracellularly. The work presented in this Thesis has therefore been focused on quantitative aspects of peptide internalization, as this provides a direct link to succeeding intravesicular peptide concentrations. Further, the endocytic events as such were studied with the aim of elucidating details of components and pathways important in the internalization and accumulation of Aβ. I propose that such mechanistic understanding could allow better understanding of early events in disease development, and that the identification of uptake modulatory components and processes could potentially be important for the development of disease-modifying treatments aimed at reducing intraneuronal accumulation.

3 Methodology

3 Methodology

This section provides a brief description of the main techniques used throughout this work. For more in-depth descriptions and further reading I refer to the referenced textbooks about fluorescence spectroscopy [195], confocal microscopy [196] and flow cytometry [197, 198]. This section also provides an outline of the approaches used to perturb endocytosis in cells.

3.1 UV-vis spectroscopy

UV-vis spectroscopy are methods that are based on the interaction of light and matter. Light, or electromagnetic radiation, can be described as a wave with an electric and a magnetic field that oscillate perpendicular to each other as well as to the direction of propagation. Light can, however, also be described as an energy package, a photon, which has particle-like properties. This is known as the wave-particle duality of light. When light interacts with matter the photon can be absorbed by the molecule (absorption spectroscopy) so that it reaches an excited state, and the excited molecule can later emit a photon (fluorescence spectroscopy).

3.1.1 Absorption spectroscopy

When light interacts with molecules, they can absorb the energy of the photon and thereby reach an excited state. For this to occur, the energy difference between the ground state and the excited state (ΔE) has to be identical to the energy of the photon, as explained by Bohr's frequency condition (Equation 1), where h is Plank's constant, v is the frequency of light, c is the speed of light and λ is the wavelength of light.

$$\Delta E = h\nu = \frac{hc}{\lambda} \tag{1}$$

If the incident light has a wavelength that fulfils this criterium, it can hence be absorbed. The energy gap for molecules to become excited correspond to electromagnetic radiation in the ultraviolet-visible (UV-vis) light range, i.e. ~ 200-800 nm. The absorption (A) of a sample at a specific wavelength (λ) can thus be analysed by probing the intensity of light before (I_0) and after (I) interaction with the sample, according to Equation 2.

$$A(\lambda) = log\frac{I_0(\lambda)}{I(\lambda)} \tag{2}$$

Absorption can also be described by the Beer-Lambert law (Equation 3), which gives the relationship between the absorption of a sample and its concentration (c) of the absorbing molecule.

$$A(\lambda) = \varepsilon(\lambda)cl \tag{3}$$

In this equation ε is the molar absorption coefficient and l is the pathlength of the sample. The Beer-Lambert law thus allows for concentration determinations of molecules in solutions, making absorption spectroscopy a powerful, yet simple, technique. Absorption spectroscopy

measurements are made in a spectrophotometer (Figure 17). The set-up contains a light source and a monochromator that determines which wavelength of light that reaches the sample. The light is then split by a beam splitter, one part passing through the sample to reach the sample detector (I), and the other part being bypassed the sample to reach the reference detector (I_0). Absorbance is typically not measured only at one wavelength, but scanned across a range of wavelengths, thereby building up the absorption spectrum of the sample.

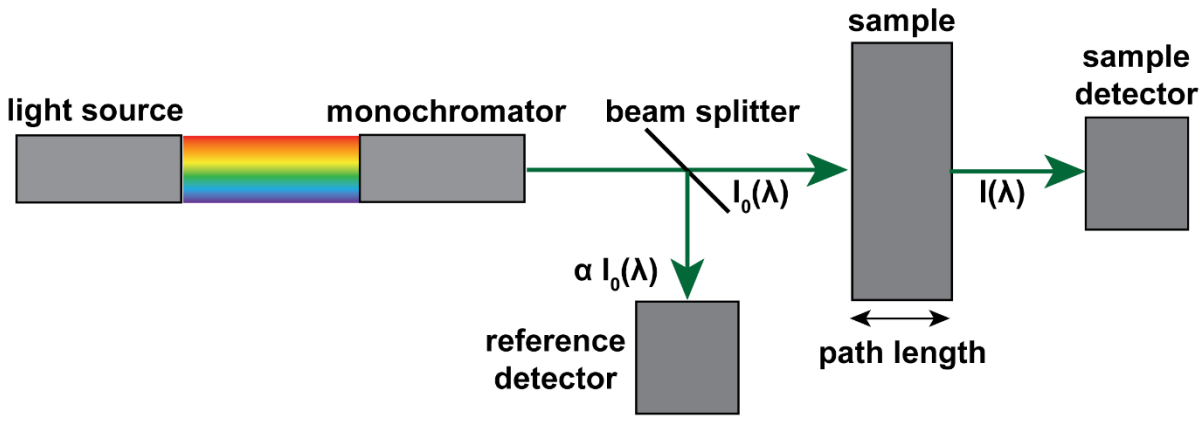

Figure 17. Set-up of a spectrophotometer used for absorption spectroscopy measurements.

3.1.2 Fluorescence spectroscopy

When molecules are excited from their ground state (S_0), via light absorption, they can reach different excited states (S_n, where $n > 0$) depending on the molecule and the energy of the photon. Further, different vibrational levels of the exited state can be reached. From the excited state, which is energetically unfavourable, the molecule will return to its ground state and this can occur via radiative or non-radiative processes, as can be displayed by a so-called Jablonski diagram (Figure 18, simplified version without phosphorescence which is not relevant for this Thesis). First, vibrational relaxation is a very fast process, so the return to the ground state will occur from the lowest vibrational level of the excited state. For most molecules, the return to S_0 then occurs via internal conversion (if excited to S_n where $n > 1$) followed by non-radiative relaxation. However, some molecules are fluorophores, meaning that they can return to the ground state via the emission of a photon that matches the energy gap between the excited state and the ground state.

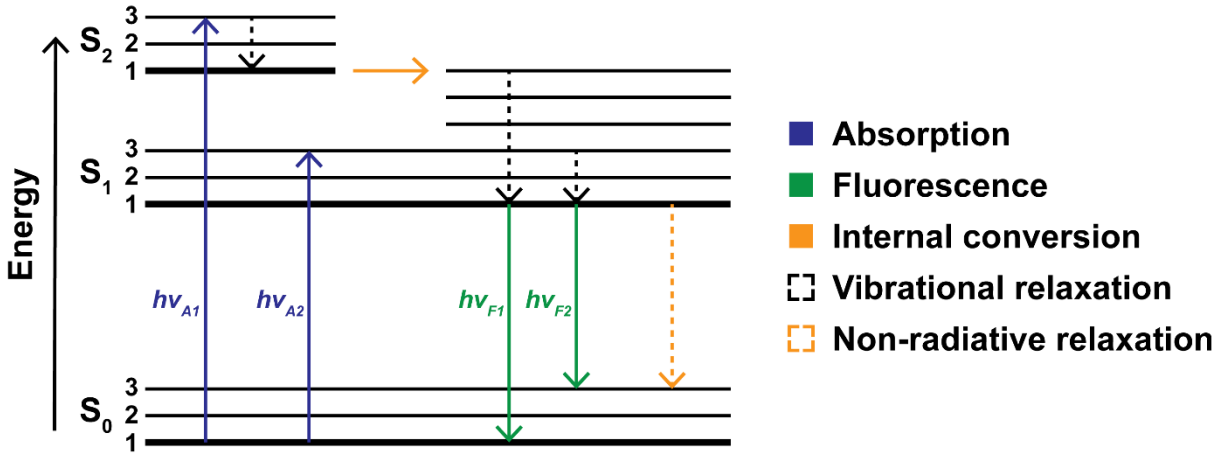

Figure 18. Jablonski diagram displaying absorption and subsequent return to the ground state via non-radiative and radiative (fluorescence) processes.

Fluorescence results in the emission of a photon which is typically of lower energy than the absorbed photon, i.e. red-shifted. This is both explained by the fact that emission occurs from the lowest vibrational level of the excited state and can return the molecule at a higher vibrational level of the ground state, and by Kasha's rule. This rule states that emission of a photon occurs from the lowest excited state (S_1), meaning that the molecule will before fluorescing first reach this level by internal conversion and vibrational relaxation. By comparing the absorption spectra and the emission spectra of a fluorophore this energy difference will be visible through the Stokes shift, the difference in wavelength between the absorption and emission maxima of the electronic transition.

Fluorescence is recorded by a fluorometer (Figure 19), but also used as detection method in many other instruments. Here, in similarity to the absorption measurements performed on a spectrophotometer, a monochromator is used to determine which wavelength of the light source that reaches the sample. When measuring fluorescence, the emitted light is, however, typically detected at a 90° angle from the incident light, which is to prevent excitation light from reaching the detector. The emitted light is passed through a second monochromator before it reaches the detector. By scanning the wavelength of emitted light an emission spectrum at a fixed excitation wavelength can be recorded. Similarly, an excitation spectrum can be recorded by varying the wavelength of excitation light while detecting the emitted light at a fixed wavelength.

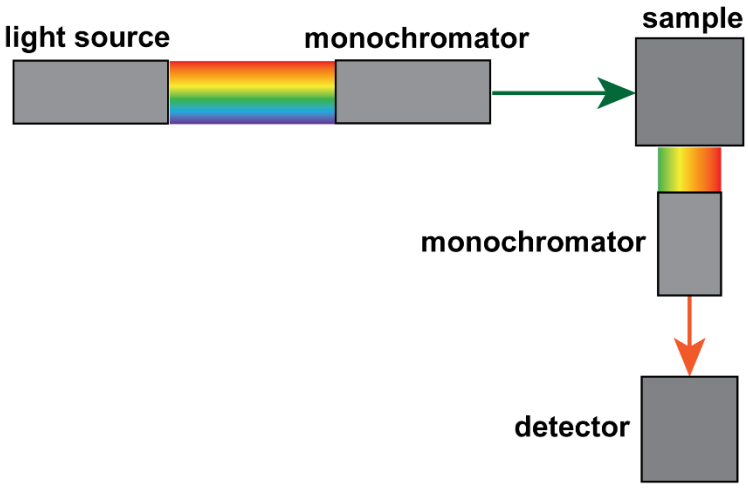

Figure 19. Set-up of a fluorometer used for fluorescence excitation and emission measurements.

The body of work contained in this Thesis is based on the use of fluorescently labelled molecules, both amyloidogenic peptides and proteins, but also other fluorescent molecules and cellular markers. The fluorescent properties of these molecules are essential for their use in the other two main techniques applied in this Thesis, confocal microscopy and flow cytometry, which will be described in the following sections.

3.2 Confocal microscopy

Microscopy is a collective name for techniques used to view objects that are too small to be seen by the naked eye. Optical, or light, microscopy uses the interaction of light with matter to visualize an object of interest. The magnified image is achieved by passing light, that is either transmitted through or reflected from, the sample through an objective, which in turn contains several magnifying lenses. The resolution (*d*) of microscope is defined as the shortest distance between two points in the sample that can be observed as separate entities, i.e. resolved. This is defined by the Rayleigh criterion (Equation 4);

$$d = 0.61\frac{\lambda}{NA} \tag{4}$$

where λ is the wavelength of the light used to visualize the sample, and *NA* is the numerical aperture of the objective. The numerical aperture can be described as the ability of the objective to collect light and is defined by Equation 5, where *n* is the refractive index of the immersion medium between the objective and the sample (e.g. 1.52 for oil), and Θ is half the angle of the cone of light that can be collected by the objective. As an example, for a sample excited with 488 nm light and detected through a 1.4 NA objective, the resolution is ~ 200 nm.

$$NA = n\sin\Theta \tag{5}$$

In fluorescence microscopy, fluorophores are used to visualize the sample. Fluorophores are excited by a light source, a lamp or a laser depending on the system. By attaching, either covalently or non-covalently, fluorophores to various biomolecules, it is possible to selectively label and image specific structures of the cell. Further, several spectrally resolvable fluorophores can be used in combination to label different structures of the cell, making it possible to perform colocalization analyses.

Confocal microscopy, or confocal laser scanning microscopy (CLSM), has been widely used throughout the work presented in this Thesis. A simplified view of the setup of a standard confocal microscope is shown in Figure 20. The basic principle of CLSM is that the sample is excited point-by-point by scanning the point of illumination across the sample while simultaneously detecting emission, thereby building up the image. The excitation source is a laser and its light is focused onto the point of illumination by first passing through a pinhole and then through the objective. Depending on the light path of the specific instrument it might also (as in the setup displayed in Figure 20) first be reflected by a dichroic mirror, which reflects light below certain wavelengths (excitation light), and transmits those above (emission light). The emitted light then passes through the same objective, and the dichroic mirror, to reach the detector. To exclude light that origins from outside the point of focus, both in xy but also above or below (z), emitted light must pass through a second pinhole before reaching the detector. This makes is possible to only image the so-called focal plane. By sequentially imaging different focal planes, a three-dimensional reconstruction of the specimen can be made.

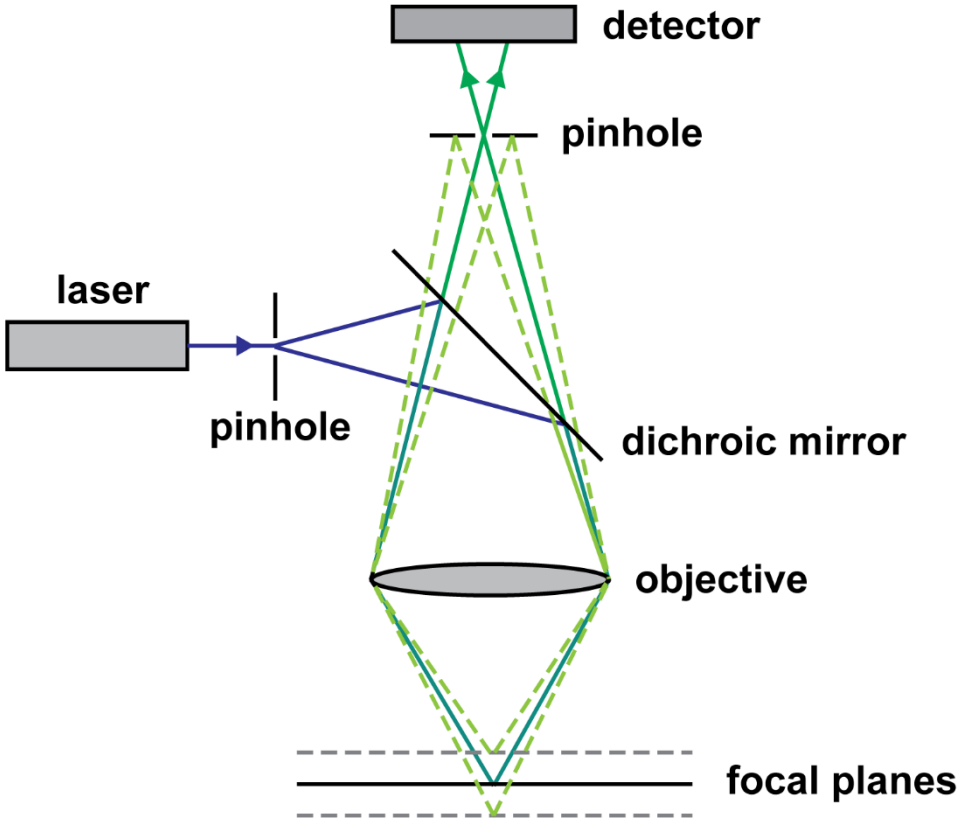

Figure 20. Set-up of a confocal laser scanning microscope.

To reach confocality, emitted light reaching the detector is strongly reduced by the use of the pinhole. Therefore, a high-intensity excitation source (laser) must be used, and the sample has to be efficiently labelled. The use of a powerful excitation source means that photobleaching and phototoxicity has to be considered, especially in live cell imaging when the sample is imaged repeatedly and/or over extended periods of time. Further, the speed of acquisition can be of considerable importance in live cell imaging, especially when imaging dynamic structures, such as the endolysosomes of interest in this Thesis.

3.2.1 FRET imaging

Fluorescence resonance energy transfer (FRET) is a process during which an excited fluorophore (called donor, D) instead of emitting a photon, transfers its excitation energy to another fluorophore (acceptor, A) through dipole-dipole coupling (Figure 21). In order for this to occur, the energy transferred from the donor has to match the transition energy of the acceptor to reach the excited state. The rate of this process is inversely proportional to the sixth power of the distance between the donor and the acceptor, thus highly distance dependent and typically only occurs if the donor and acceptor are less than 10 nm separated in space [199].

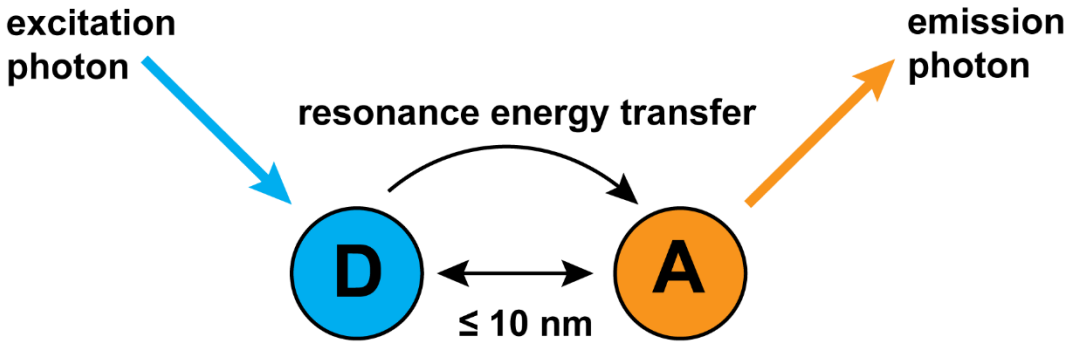

Figure 21. Fluorescence resonance energy transfer (FRET) from an excited donor (D) to an acceptor (A) molecule.

Due to the close packing of individual peptide molecules in amyloid fibrils, it is possible to follow protein aggregation via FRET analysis of fluorescently labelled monomers [165, 200]. When microscopy is applied to study the occurrence of FRET, this is known as FRET imaging [199]. FRET analysis of images acquired by confocal microscopy was used in **paper III** to probe for the evolution of a FRET signal, indicative of peptide aggregation, from fluorescently labelled Aβ internalized into endolysosomal vesicles.

3.3 Flow cytometry

Flow cytometry is an analysis method that provides information of cellular features on a cell-by-cell basis. Common applications include identification of different cell types within a heterogeneous population, quantification of the cellular DNA content, measurements of intracellular pH, and quantification of fluorescent probes on the surface or in the interior of cells. Flow cytometry was used throughout this Thesis to quantify the cellular uptake of fluorescently labelled proteins and peptides, as well as to analyse cells transfected with plasmids encoding for fluorescent proteins conjugated to a protein of interest.

A schematic presentation of a flow cytometer is depicted in Figure 22A. A dispersion of the cells to be analysed is continuously injected into a flow chamber. A prerequisite for flow cytometry is, however, that the cells are analysed one-by-one, and to achieve this, the cells are focused prior to analysis. In many instrumental setups this is achieved by subjecting the sample flow to a sheath flow, which flows in a laminar fashion around the sample flow and focuses the beam. This is called hydrodynamic focusing. A less conventional way of sample flow is instead by driving the sample through a microcapillary flow cell (Figure 22B). Due to the narrow dimensions of the flow cell, the cells are forced to pass the detector one-by-one. The instrument applied in the work conducted within this Thesis was equipped with a microcapillary flow cell.

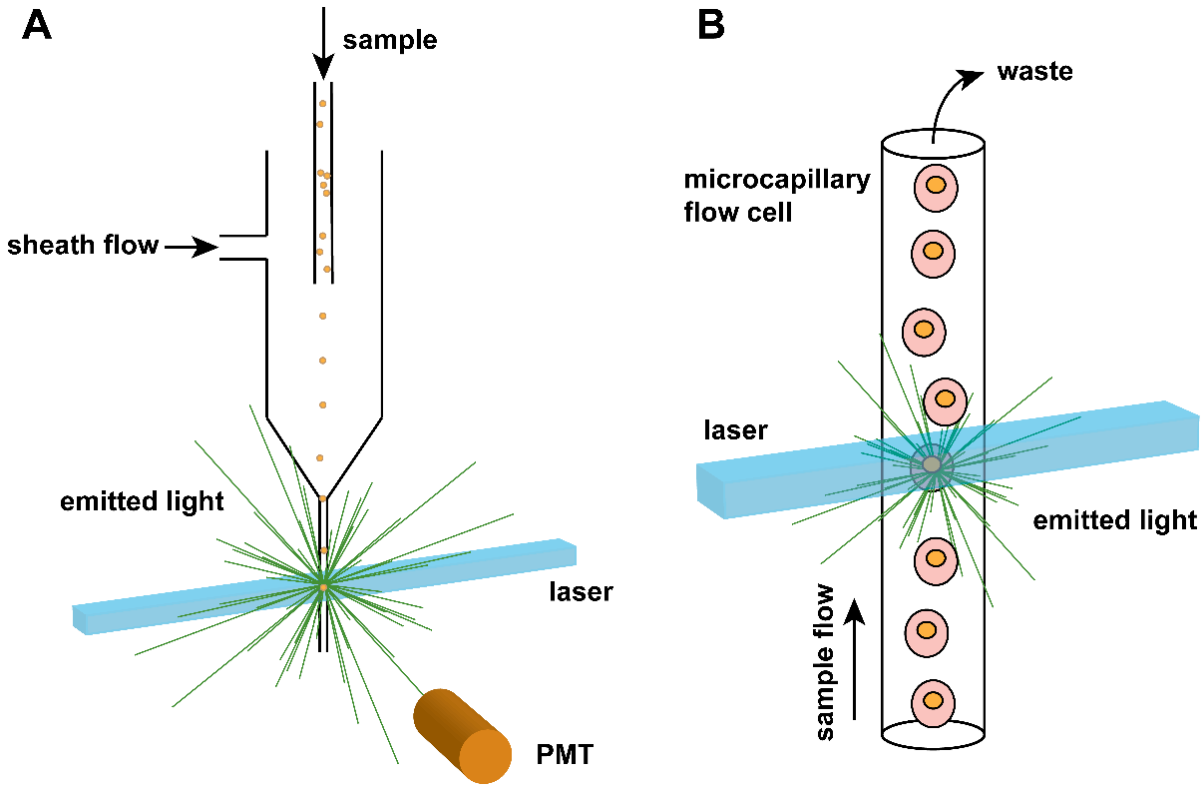

Figure 22. Set-up of a flow cytometer with (A) a conventional sheath flow-based flow cell, and (B) the microcapillary flow cell used in this Thesis.

The focused stream of cells passes one or several laser beams, from which the cells will scatter light, and by which fluorescent cell markers can be excited. Scattered light is detected in the forward (10° from direction of laser beam) and side (90°) direction. Since cells in suspension are generally spherical in shape, the intensity of forward scatter gives a measure of the size of the cells. The side scatter, on the other hand, will depend on how the cells scatter light intracellularly, and hence the intensity of side scatter gives a measure of the granularity of cells. Depending on, for instance, viability and cell type, cells will display varying scattering properties. In a so-called forward versus side scatter plot, or FSC/SSC dot plot, where each particle passing through the laser beam is displayed in terms of its scattering properties, cell populations will therefore appear as clusters of detected events (Figure 23A). Debris in the sample will typically appear at low forward scatter due to the small size of these particles compared to cells, and clusters of cells will appear at larger forward scatter compared to monodispersed cells. Apoptotic or dead cells will appear in a different region compared to their live counterparts, and this means that, as long as the location of dead and live cells have been determined previously with the same instrument settings and using live/dead fluorescent markers, the general viability of the cell population can be studied without the use of these fluorescent markers, freeing spectral regions for other cellular markers of interest.

If cells have been stained with fluorescent markers, these can be excited by the laser beam and their emission detected, generally through the use of photomultiplier tubes (PMTs) located after suitable bandpass filters. In the ideal case, the emission intensity correlates linearly to the amount of fluorophore labelling of a cell, which makes quantitative comparisons between samples possible. The fluorescence intensity of analysed cells is usually either displayed as a histogram (Figure 23B) or, if labelled with multiple fluorescence markers, as a dot plot. In this Thesis the average intensity of analysed cells was generally used as a measure of peptide uptake, allowing for comparisons between different samples and treatments.

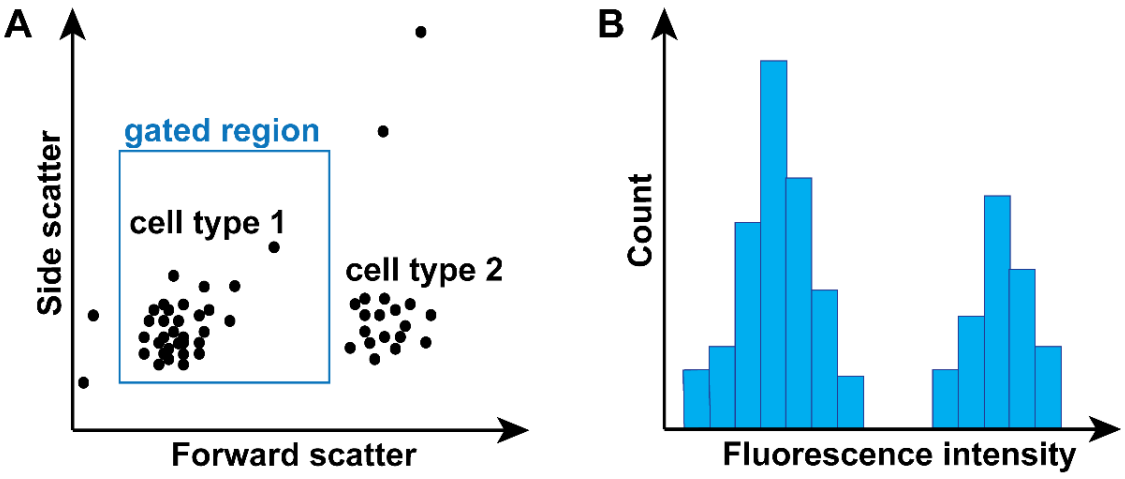

Figure 23. Typical data obtained from flow cytometry measurements; (A) scatter plot of FSC/SSC and (B) histogram of the intensity distribution of analysed cells.

3.4 Perturbation of endocytosis

All papers presented in this Thesis deal with endocytosis of amyloidogenic proteins. There are several different approaches that can be taken to map key players in endocytic uptake of a molecule of interest and this section intends to give a brief overview of the type of experiments performed within this Thesis.

First, to verify that a molecule indeed enters cells via endocytosis, a general block of active uptake processes can be performed by either low temperature incubation at 4 °C [201] (**paper I** Aβ, **paper II** α-syn) or by depleting cells of ATP [202] (**paper I**, Aβ). Low temperature incubation changes the fluidity of the plasma membrane, which could influence how the molecule interacts with, and potentially crosses over, it. ATP depletion is therefore a good complementary approach, although it is a more perturbing treatment often reducing cell viability and thereby resulting in potential off-target effects. Osmotic swelling via exposure to hypotonic medium is another approach that generally stops all endocytic processes [203, 204] and that was applied in **paper IV** with Aβ, where cells were also exposed to a sequence of hypotonic-isotonic conditions to probe for involvement of the CLIC/GEEC pathway.

Endocytic uptake can also be studied by examining how the molecule interacts with certain cell surface components. This could be applied in a specific manner by studying the involvement of certain receptors. In this Thesis, this was however approached in a relatively general manner by studying the involvement of cell surface proteoglycans (**paper III**, Aβ), by various washing procedures prior to analysis, and pre-treatment with enzymes (in this case heparinase) that degrades specific components of the cell surface (**paper II**, α-syn).

To analyse the contribution of specific endocytic paths, main approaches can be divided into the exposure to pharmacological inhibitors of endocytosis, or transfection and expression of dominant active (DA), wild-type (WT), or dominant negative (DN) variants of endocytic components. siRNA could also be used but was not applied within the work presented in this Thesis. A range of pharmacological inhibitors (chlorpromazine, dynasore, IPA-3, EIPA, wortmannin, cytochalasin A and D) were applied in **paper I** for studies of Aβ. These inhibitors in theory have a straightforward application, but much due to their often narrow treatment window and varying response between cell lines, their use requires extensive optimization and careful controls to make sure that the observed effects are indeed specific to a reduced endocytic uptake, rather than due to non-specific toxicity effects. Also, exposure times are limited, thereby also limiting the possible incubation time with the molecule of interest, which can in turn pose challenges in terms of uptake signal. In addition to their ease of use, another benefit with the pharmacological approach is, however, that all cells in a sample are exposed to the same inhibitor concentration. This is in contrast to the transfection approach where not all cells are transfected and, further, where transfected cells can have varying levels of inhibitory protein expression.

The transfection approach was applied in **paper I** (Aβ; AP180-C, dynamin, Arf6), **II** (α-syn; AP180-C) and **IV** (Aβ; Rho GTPases Cdc42, Rac1 and RhoA). In these experiments, the expressed protein was conjugated to a fluorescent protein, thereby allowing for discrimination of successfully transfected cells, as well as offering the possibility of correlating the analysis of uptake levels to that of protein expression (e.g. the inhibitory dose). In addition, a major benefit with this approach is the lower risk of off-target effects. Secondary effects resulting from long

periods of protein expression, or high expression level must, however, still be considered. Here it can also be added that some components, for example the RhoA GTPases studied in **paper IV**, are important in several different endocytic mechanisms, and their response should therefore be seen more as a mapping type of analysis, rather than probing for the exact involvement of specific uptake paths.

Lastly, the use of proper controls is a requirement for endocytic uptake experiments to be reliable, considering the already mentioned potential pitfalls of off-target and secondary effects. This is, however, complicated by the fact that, among other things, not all endocytic paths have well-defined specific ligands, and CIE is far from fully understood. To tackle this, the approach applied in this Thesis was to analyse the uptake of the CME-ligand Trf [135] and the fluid-phase marker dextran 10 kDa [205], and when possible probe for the mechanism of interest from multiple angles.

4 Original work

4 Original work

This chapter presents a summary and discussion of the most important results from the research papers (**paper I**-**IV**) included in this Thesis. It is structured so that relative quantitative comparisons of cellular uptake depending on properties of the peptide itself (isoform of Aβ and length of α-syn fibrils) as well as on the presence of proteoglycans on the cell surface are summarized in the first two sections, followed by mapping of endocytic paths and key players in monomeric Aβ uptake in the third section.

4.1 Quantification and comparison of cellular uptake of amyloidogenic proteins

This section describes my work on quantification of cellular uptake of Aβ(1-40) and Aβ(1-42) (**paper I**) as well as on the uptake of α-syn amyloid fibril preparations of different average lengths (**paper II**). The Aβ(1-40) isoform is naturally of higher abundance than the longer 42 amino acid residues isoform [11]. Still, Aβ(1-42) is found to accumulate to a higher extent in intraneuronal locations [12] and in plaque deposits [95]. By studying quantitative aspects of cellular uptake of the two isoforms, the aim was to elucidate potential uptake details of importance for this distinction and its pathological consequences. Amyloid fibril fragmentation has also been demonstrated to result in higher cellular toxicity [176], and by studying how α-syn fibril fragmentation relates to cellular uptake, the aim was to elucidate whether fragmentation-related toxicity could be related to intracellular accumulation following uptake.

4.1.1 Uptake of monomeric Aβ(1-40) and Aβ(1-42)

My research has primarily focused on understanding how monomeric forms of the Aβ peptide are internalised into cultured cells, and as such it was important to establish methods that allowed robust characterization and quantification of their uptake. My work on monomeric Aβ uptake complements cellular uptake characterizations of oligomeric [164] and fibrillar [206, 207] species. I first adapted a protocol commonly used to solubilise unlabelled synthetic Aβ peptides to the fluorescently labelled Aβ(1-40) and Aβ(1-42) peptides used in my Thesis. The protocol involved pre-treatment with hexafluoro-2-propanol (HFIP) to dissolve pre-formed aggregates [208]. This method was found to be very successful, providing highly monomeric preparations (Figure 24A) that, furthermore, did not aggregate in bulk at the concentrations used and for the timespan of our typical experiments. This protocol was subsequently used in **paper I**, **III** and **IV**. I will hereafter denote peptides dissolved this way as monomers even if it cannot be excluded that a small, non-detectable by the methods I have used, oligomer fraction would exist, or immediately form, upon addition to the cells. When applied to SH-SY5Y cells, Aβ rapidly internalised into distinct puncta (Figure 24B), which in itself is strongly indicative of endocytic uptake as will be further elaborated in section 4.3. The protocol for Aβ preparation was of crucial importance for minimizing the risk that residues of pre-formed Aβ oligomers or

other aggregates influences the result. Further, there has been contradicting results in the field depending on what specific preparation protocols and hence types of oligomers that have been studied [209], emphasizing the importance of well-characterized starting material.

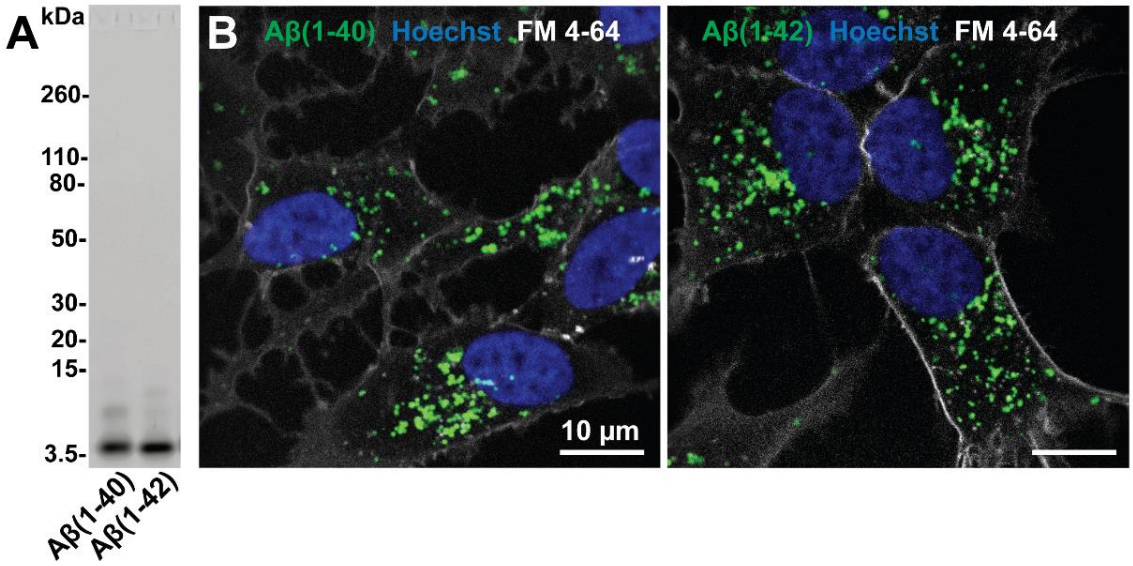

Figure 24. (A) SDS-PAGE of Aβ(1-40) and Aβ(1-42) solutions and (B) cellular uptake of the Aβ peptides after 24 hours of incubation.

In **paper I,** I compared the uptake of Aβ(1-40) and Aβ(1-42) into cultured cells, using fluorescence imaging and flow cytometry approaches to quantify their relative degrees of accumulation. The results display consistently in the range of twice as much Aβ(1-42) internalisation compared to the shorter Aβ(1-40) isoform (Figure 25). This is true both over a range of peptide concentrations (Figure 25A), as well as over time (Figure 25B), and agrees well with what I later observed also in CHO-cells (**paper III**), as well as with previous observations by Burdick et al [210] at higher Aβ concentrations. The two-fold difference in uptake is apparent already after < 2 hours of incubation, and is stable for at least three days. Interestingly, the cell uptake does not seem to saturate, but rather display a near linear profile of Aβ accumulation, suggesting that cells have a very high capacity of accumulating Aβ which could be a combination of effective internalization and impaired clearance (for example due to aggregation). I also showed that the difference in uptake of Aβ(1-40) and Aβ(1-42) was not dependent on the fluorescent tag (HiLyte Fluor488), as similar results were also obtained with peptides labelled with a red fluorophore (HiLyte Fluor647). Considering the relatively low natural abundance of Aβ(1-42) compared to Aβ(1-40) [84], these differences in extent of cellular internalisation could offer one explanation to the preferential cellular accumulation of Aβ(1-42) [12, 210].

In the experiments presented in this Thesis, the applied Aβ concentration has typically been 0.5-2 µM, to yield sufficiently strong signals for detection. Previous studies have demonstrated that Aβ can indeed aggregate intravesicularly in cultured cells after incubation in this setting [15, 16]. The extracellular Aβ concentration *in vivo* is, however, much lower (< 10 nM [15, 211]), although local variations in peptide concentration are likely to exist. To evaluate

cellular uptake and accumulation as a concentrating machinery, I also set out to determine the intravesicular Aβ concentration after peptide uptake. Using a microscopy-based approach I measured the number of Aβ molecules per cell after incubation in 1 μM peptide solution for 8 hours to be ~ 400,000 for Aβ(1-40) and ~ 800,000 for Aβ(1-42) (Figure 26), thus agreeing well with the flow cytometry results in Figure 25. Taking into account the cellular volume of lysosomes [212], this was then estimated to correspond to a ~ 100x concentrating factor (60 μM Aβ(1-40) and 140 μM Aβ(1-42)), demonstrating the powerful effect of intravesicular accumulation, and agreeing well with what has previously been reported by Hu et al [15]. The high concentrating potential of endosomes is interesting in relation to Aβ aggregation; especially in light of the observations that we observe Aβ accumulation to be virtually non-saturable, meaning that this factor could build up to be significantly higher with time, theoretically allowing low abundance extracellular Aβ to be concentrated and aggregated in endolysosomes also at very low extracellular peptide concentrations.

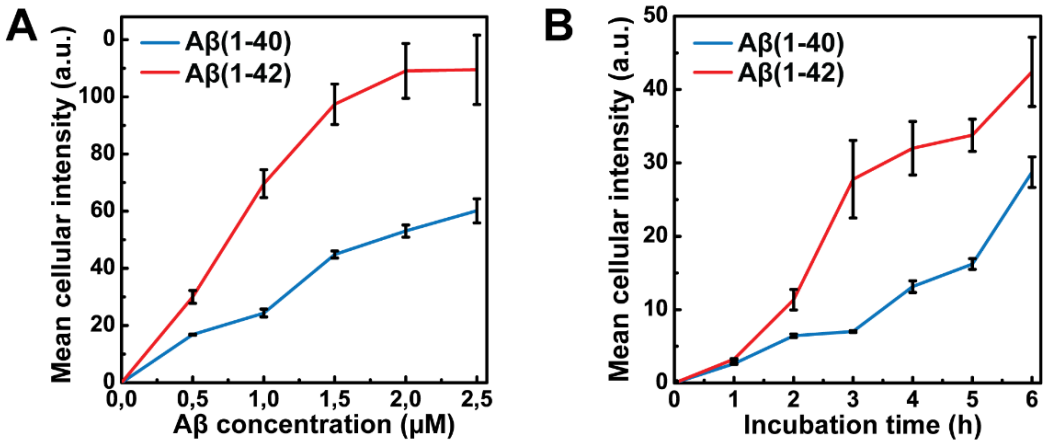

Figure 25. Relative quantification of Aβ(1-40) and Aβ(1-42) uptake in SH-SY5Y cells. Flow cytometry analysis of HF488-labelled Aβ accumulation at varying (A) peptide concentration and (B) incubation time.

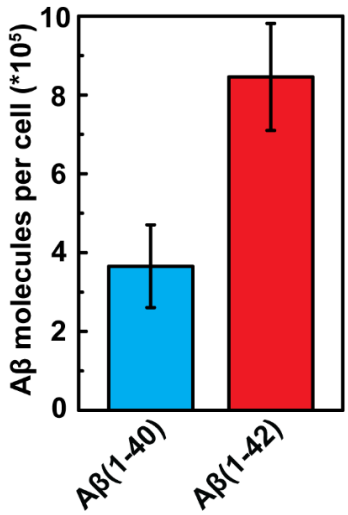

Figure 26. Microscopy-based absolute quantification of Aβ accumulation in SH-SY5Y cells.

4.1.2 Influence of α-syn fibril length on endocytic uptake and toxicity

Another characteristic of amyloid forming proteins that I have set out to study in terms of cellular uptake was the effect of fibril length; the study presented in this Thesis considers α-syn (**paper II**), although attempts were also made with fragmented forms of Aβ(1-42) fibrils (which were found to be prone of forming clumps, possibly due to a higher ability of lateral association). α-syn was selected due to its demonstrated enhanced cellular toxicity of fragmented fibrils compared to their full length counterparts [176]. We prepared fluorescently labelled α-syn fibrils of different lengths by mixing unlabelled and HiLyte Fluor488-labelled α-syn monomers followed by fragmentation of the resulting fibrils. We exposed the α-syn fibrils to either stirring for extended periods of time (up to 11 hours) or sonication; this yielded a range of fibril samples with an average length as low as ~ 100 nm (Figure 27). The fibril thickness was not influenced by the fragmentation process, demonstrating that the resulting fibrils did not associate laterally.

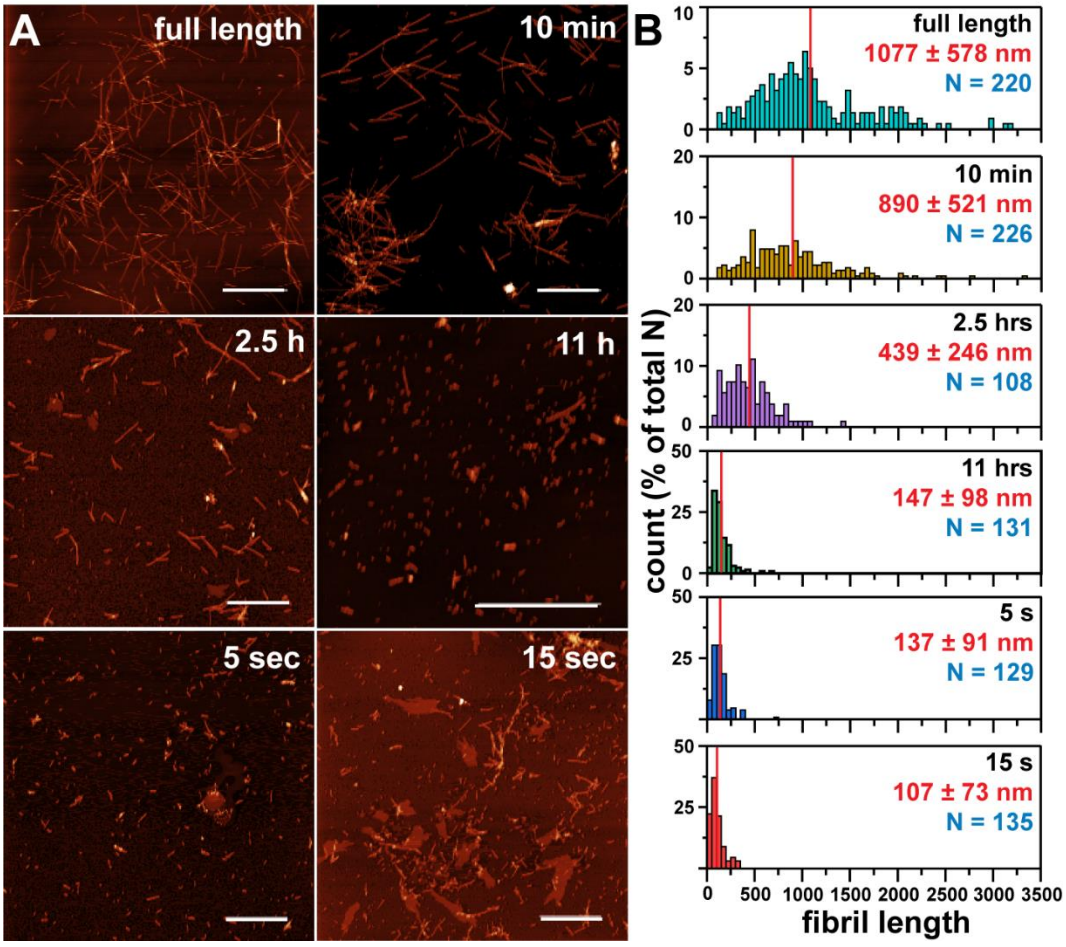

Figure 27. Morphological characterization of fragmented α-syn fibrils. (A) AFM images and (B) resulting length distributions of fragmented fibrils. 10 min – 11 hours denotes time of fragmentation by stirring, and 5 – 15 sec by sonication. The scalebar in (A) is 2 µm.

I quantified the cellular uptake of these different α-syn fibrils by flow cytometry based on the intensity of the internalised protein, similarly as described for the Aβ monomers. It was

therefore of high importance that the fluorescence emission signal was directly corresponding to protein concentration. When amyloid fibrils containing fluorescently labelled protein are formed, there is a possibility that the emission of the sample can decrease due to self-quenching in these highly ordered structures. This has been observed previously in our research group with Aβ [16]. I therefore measured emission intensity of the monomers and the different fibrillar α-syn samples and could confirm that in this case their emission intensity was within error identical. The fibrils were 50 % labelled, based on the fraction of fluorescently labelled monomer added to the aggregation reaction. This was to interfere as little as possible with fibril assembly, but it can also be assumed that the risk of self-quenching in fibrillar samples would be higher if the labelling percentage would have been increased.

To characterize the uptake of the fragmented α-syn fibrils into SH-SY5Y cells, I performed confocal imaging and flow cytometry analysis. I subjected the cells to the fibrils for 3 hours at 4 °C and 37 °C (Figure 28); this resulted in extensive membrane binding at all conditions. Furthermore, the α-syn fibrils did not internalise to any measurable extent in the cells incubated at 4 °C, whereas intracellular accumulation in distinct puncta, likely endolysosomes, was observed after incubation at 37 °C. This was primarily seen for the fibril samples with an average length shorter than 500 nm, which suggested that a size cut-off in cell uptake may exist.

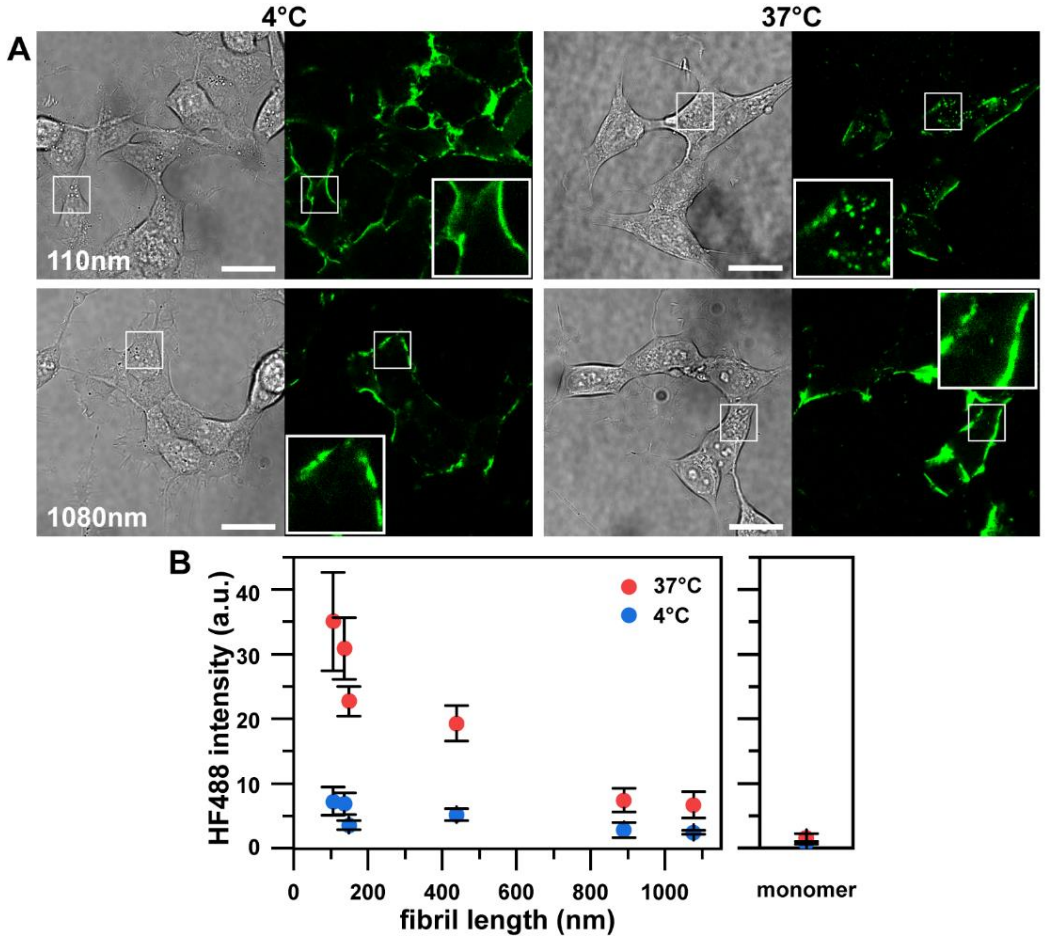

Figure 28. Cell uptake of fragmented α-syn fibrils following incubation at 4 °C or 37 °C for 3 hours, analysed by (A) confocal microscopy and (B) flow cytometry. The scalebar in (A) is 20 µm.

The uptake was also quantified by flow cytometry; here it was noted that the 4 °C incubated samples displayed very low fluorescence compared to what would be expected based on the extensive membrane binding in the imaged samples. I subsequently showed that the harvesting procedure by trypsination prior to flow cytometry analysis efficiently removed at least the majority of the membrane bound fraction suggesting that protein moieties on the cell surface is important for α-syn fibril association. I also performed longer, 24 hours, incubations with the fragmented α-syn fibrils at 37 °C (see **paper II**). The correlation between uptake and fibril length in these experiments agree with the data in Figure 28. The experiment also included more data points (from merging of data sets obtained with different fibril batches) and thereby describe the length-dependent cell uptake in greater detail. Furthermore, by comparing the fibril length distributions (Figure 27B) with the degree of uptake of differently sized samples, we could estimate a cut-off for efficient uptake at ~ 400 nm. This is interesting in relation to the size of endosomal vesicles. CCVs are typically < 200 nm [137] and the fibrils would hence need to bend to accommodate into these type of vesicles. Instead, macropinocytosis would be a more likely uptake mechanism based on the size of the vesicles formed [150]. In contrast, the α-syn monomers internalise very inefficiently, even in comparison to the long α-syn fibril variants. These observations agree with recent observations by Hoffman et al [213] and demonstrates how differently monomeric Aβ and α-syn behave in terms of cellular uptake.

As already mentioned, fragmentation of amyloid fibrils has been correlated to toxicity [176, 214]. In **paper II** we performed toxicity experiments using the MTT reduction assay (Figure 29) and could show that toxicity correlates to cellular uptake, which in turn is correlated to average fibril length; shorter α-syn fragments display higher cell uptake and hence also toxicity. We therefore postulate that the mechanism of extracellular α-syn fibril toxicity originates intracellularly via endocytic uptake.

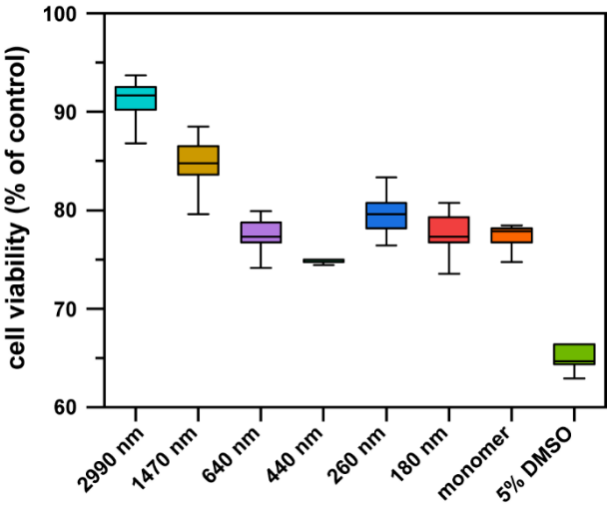

Figure 29. Toxicity of α-syn fragmented fibrils measured by the MTT assay.

To summarize, **paper I** and **II** consider uptake of monomeric Aβ and α-syn, as well as fibrillar α-syn. In a longer term perspective, a full and comparative characterization of the uptake and accumulation of both proteins and their various aggregated forms would be valuable, but it was

not possible to complete this within the timeline of this Thesis. As we found that the fibrillar forms of Aβ and α-syn display highly different behaviours, the fragmentation studies were better suited for α-syn; attempts to study fibrillar forms of Aβ were made, but fibrils did not form discrete fibril fragments, but rather clumps, after fragmentation. In addition, fragmented Aβ fibrils were observed to form diffuse bigger aggregates upon application to the cells, and further characterization as well as protocol development would be needed. Different variants of oligomers are also interesting in terms of uptake studies, based on their observed cellular toxicity [209, 215], but have not been in focus of this Thesis.

4.2 Proteoglycans in cellular uptake of Aβ and α-syn

To better understand the molecular prerequisites for the uptake of Aβ and α-syn described in section 4.1, I next turned to study the influence of a property of the cells themselves – the presence of proteoglycans on their cell surface – for uptake of these species, as outlined in **paper II** and **III**, with a focus on Aβ(1-42) monomers.

4.2.1 Proteoglycans in Aβ uptake

In **paper III**, I studied the effect of cell surface proteoglycans (PGs) on the uptake of monomeric preparations of Aβ(1-40) and Aβ(1-42) by comparing two different cell lines; wild-type CHO-K1 cells and the mutant cell line CHO-pgsA-745, which lacks all protein-attached heparan and chondroitin sulfate chains [216]. These two cell lines have previously been used to show that uptake of Aβ monomers after long incubation times (24 hours) depends on PGs and that lack of PGs results in diminished Aβ toxicity [188-190].

However, before quantifying the uptake of Aβ in the two different CHO-lines, I first characterized their endogenous endocytic rate by measuring the uptake of the fluid phase-marker dextran 10 kDa (Figure 30). This is important as cells can have highly varying levels of endocytic activity (a fact that I also demonstrated in **paper I**, comparing Aβ and dextran uptake in three cell types). A difference in Aβ uptake between the CHO-lines could thus be partially explained by this, rather than by specific effects arising from the interaction with PGs. Importantly, I found the K1 cells to consistently internalize approximately twice the endocytic volume compared to pgsA-745 cells. This is a new observation, not only important for my work, but for the general use of these cell lines to determine the effect of PGs in cellular uptake. Moreover, by quantifying the difference in endocytic rates, I could develop a novel approach to compensate quantified uptake for cell type-dependent differences in endocytic rate. Indeed, a number of studies on both cell penetrating peptides (CPPs) as well as other therapeutic peptides have demonstrated PG-dependency in the range of ~ 2 times higher uptake in K1 cells compared to pgsA-745 cells [217-220], which could in fact be effects of perturbed endocytic rate rather than specific PG-dependency. It should be noted that I have not further characterized the observed difference in endocytic rate to determine more exactly if certain endocytic paths are more affected than others. However, such characterization would be interesting as a tool to deepen understanding of PG-dependent cell uptake in general and Aβ internalisation in particular.

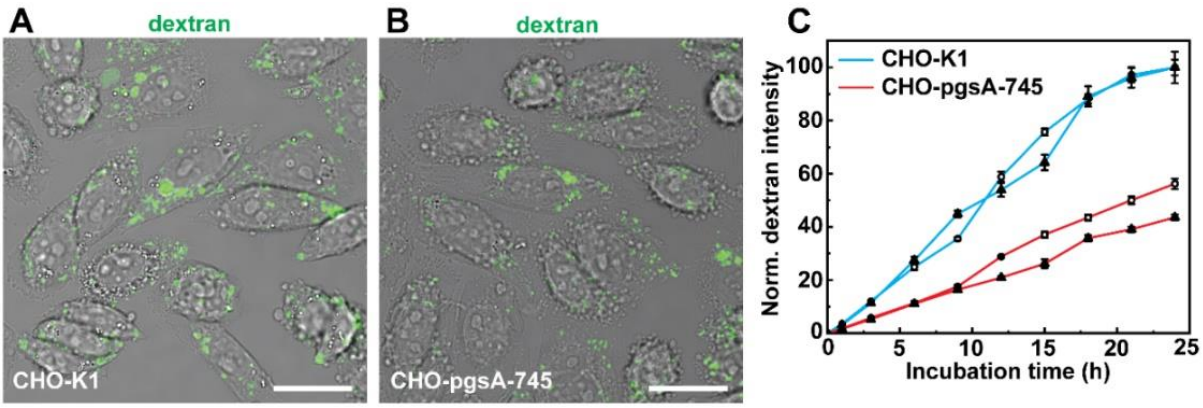

Figure 30.Uptake of dextran 10 kDa in CHO-K1 and CHO-pgsA-745 cells analysed by (A-B) confocal microscopy and (C) flow cytometry. The scalebar in (A-B) is 20 µm.

I next studied how PGs influence the uptake of Aβ(1-42) as function of time and found that after 24 hours, K1 cells internalize ~ 13 times more Aβ(1-42) than the mutant pgsA-745 cells, in agreement with previous studies [190]. After 3 hours of incubation, however, the corresponding difference was only ~ 2 times, and interestingly, after compensating for the difference in endocytic rate of the two CHO-variants the difference in Aβ(1-42) uptake after 3 hours is non-significant (Figure 31A). This is, to the best of my knowledge, the first observation that PG-dependent cell uptake can develop over time, which is interesting both in the context of Aβ(1-42) aggregation and the possibility of PG-clustering, which has been shown to stimulate endocytosis in other cases [181]. To better understand the temporal evolution of PG-dependency, I performed sets of experiments where the cells were incubated with Aβ(1-42) for 0 – 24 hours with 3 hours intervals (Figure 31B). Interestingly, the data indicate a sigmoidal increase in Aβ(1-42) uptake in K1 cells resembling growth kinetics of amyloid fibrils. However, I did not observe any aggregation of the peptide in bulk during the timespan of the experiment, which lead us to suggest that instead local aggregation at the cell surface just prior to cell uptake could explain the observed behaviour. Aβ(1-42) has in fact previously been observed to accumulate and locally aggregate at the plasma membrane prior to uptake [165]; I did, however, not observe this type of membrane accumulation for Aβ(1-42). Assumedly the uptake is such a fast event that there are, at any given moment, not a high enough number of fluorescent molecules to detect the peptide at the membrane. In work presented by Jin et al [165] cells were cooled down to block endocytosis and hence allow for detection of Aβ(1-42) at the membrane; following this protocol I observed formation of occasional Aβ(1-42) aggregates at the cell surface, but when tracking these by time-lapse imaging, I found that they never entered the cells once endocytosis was resumed (by bringing the cells back to 37 °C). One could therefore question the biological relevance of these species.

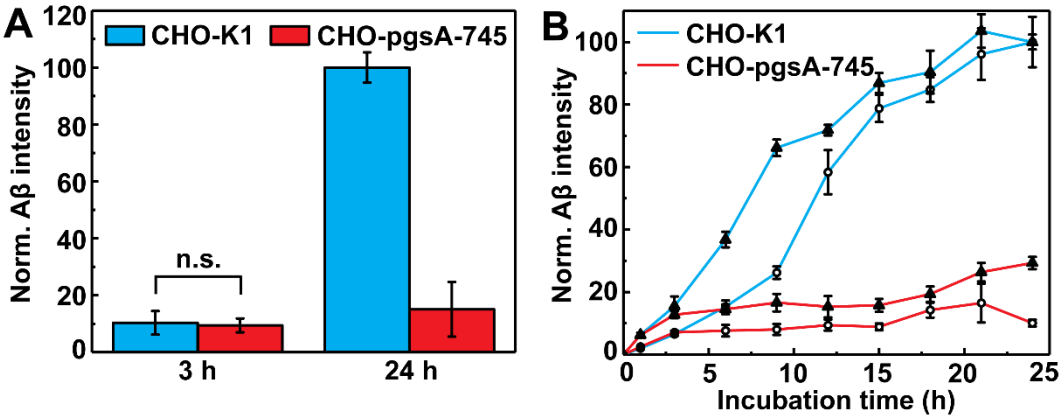

Figure 31. Uptake of Aβ(1-42) in CHO-K1 and CHO-pgsA-745 cells analysed by flow cytometry and compensated for the difference in endocytic rate (i.e. dextran uptake) between the two cell lines.

As a tool to study whether local peptide aggregation at the surface is a prerequisite for uptake, we exposed the cells to pre-formed oligomers of Aβ(1-42) (Figure 32) with the hypothesis to observe an immediate difference in uptake between the two cell lines. These oligomers were differently internalized by the two cell types after 24 hours of incubation, consistent with data on the monomer, but were also difficult to detect at short incubation times as they were formed with only 10 % labelled peptide in order not to interfere with oligomer formation. Still, we do not observe any difference between the two cell lines after 3 hours of incubation.

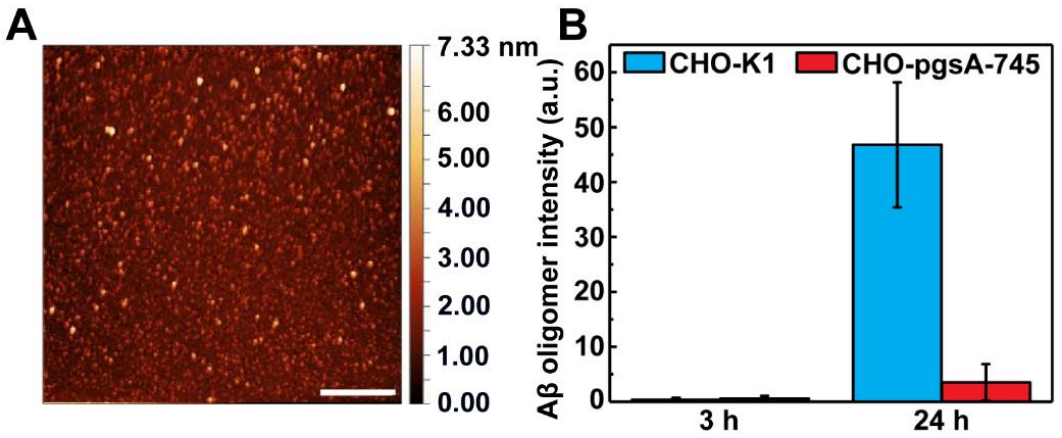

Figure 32. Morphological characterisation and uptake of pre-formed Aβ(1-42) oligomers. (A) AFM image of Aβ(1-42) oligomers. The scalebar is 1 µm. (B) Uptake of Aβ(1-42) oligomers in CHO-K1 and CHO-pgsA-745 cells analysed by flow cytometry.

Instead of further attempting to probe the aggregation of Aβ(1-42) prior to uptake, I performed FRET imaging by confocal microscopy to elucidate whether the aggregation state of the intravesicular Aβ(1-42) differed depending on if the cells where PG-deficient or not (Figure 33). From these experiments, I could conclude that although the pgsA-745 cells contained a lower number of bright puncta than wild-type cells, the intensity of bright puncta was comparable in the two cell lines. It thus seems like the difference in uptake results in the

PG-deficient cells containing fewer Aβ(1-42) positive endolysosomal vesicles rather than less Aβ(1-42) per vesicle. Lastly, the analysis of the FRET signal in relation to green signal showed no difference in the two cell lines, indicating that regardless of what occurs prior to uptake, the intravesicular Aβ(1-42) is aggregated to the same extent in both lines at the end of the 24 hours incubation period, which is reasonable if the concentration is similar in both cases.

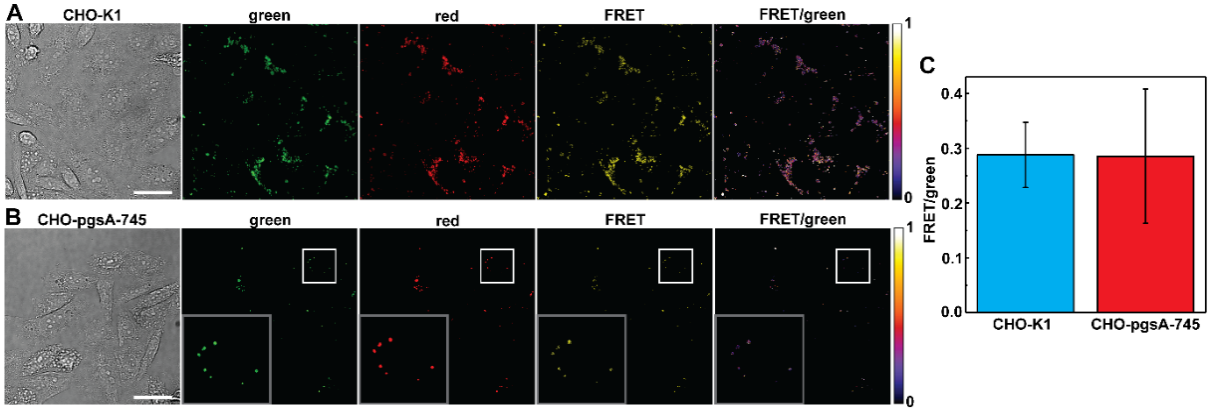

Figure 33. FRET imaging by confocal microscopy of HiLyte Fluor488 and -647 labelled Aβ(1-42) internalised into CHO-K1 and CHO-pgsA-745 cells. (A-B) Representative confocal microscopy images and (C) FRET signal in relation to green signal. The scalebars in (A-B) are 20 µm.

4.2.2 Proteoglycans in α-syn uptake and cell surface binding

I was also interested in how PGs potentially influence the internalisation of α-syn fibrils. As has already been discussed, the uptake of both α-syn monomers and long fibrils is highly inefficient, and I therefore decided to study the importance of PGs for internalisation of only short fragmented α-syn fibrils, on average 110 nm in length (**paper II**). As discussed in section 4.1.2, these species show both extensive membrane binding and are capable of being taken up by cells. Their potential dependence on PGs was thus evaluated as a way to characterize their strong membrane binding properties. Instead of using the CHO cell lines, I pre-treated SH-SY5Y cells with heparinase I/III, to cleave sulfonated heparan chains. This reduced the internalisation of α-syn fibrils in an enzyme concentration-dependent manner (Figure 34A). This supports previous findings by Holmes et al [174] that co-incubation of sonicated α-syn fibrils with heparin reduces fibril uptake, and demonstrates the importance of PGs for cellular uptake of α-syn fibrils. Interestingly, washing α-syn incubated cells with heparin did not remove the already surface-bound fibrils, illustrating their strong interaction with the cell surface. To further characterize the membrane binding properties α-syn fibrils, I analysed cells harvested by trypsination, compared to by enzyme-free cell dissociation (Figure 34B); this showed that trypsin treatment resulted in higher extent of removal of cell surface-bound α-syn species. This could be an effect of trypsin acting both on α-syn itself and on putative protein interaction partners on the cell surface.

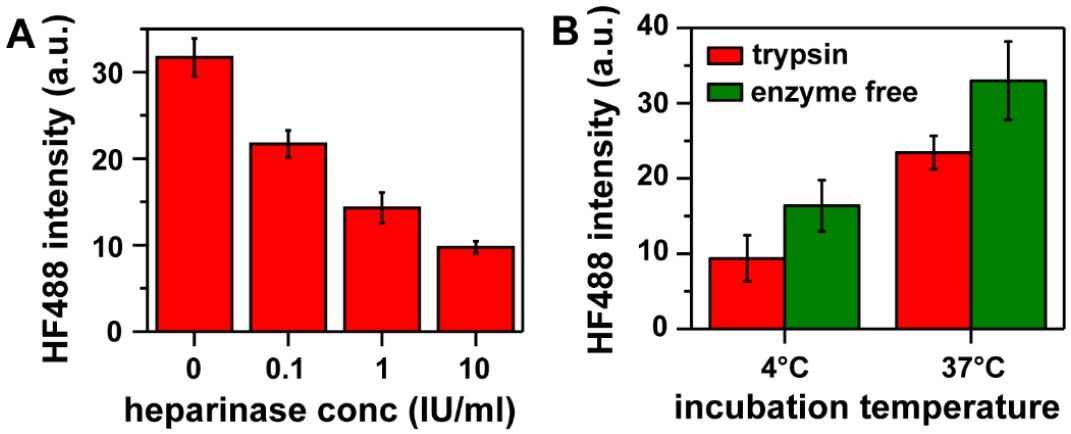

Figure 34. Cell surface binding of α-syn fragmented fibrils analysed by flow cytometry. (A) Uptake of α-syn fragmented fibrils of average length 110 nm in SH-SY5Y cells pre-treated with heparinase I/III. (B) Intensity of α-syn in cells incubated with fibrils of average length 110 nm for 3 hours and harvested by either trypsin or an enzyme-free cell dissociation buffer.

4.3 Endocytic paths in Aβ uptake

There have been several reports, including my own work, on Aβ being internalized into cultured cells by endocytosis [15, 16, 105], although there are also occasional reports on direct membrane penetration [160, 221]. Therefore, before mapping for specific endocytic paths and involvement of their respective components, I evaluated whether the Aβ peptide entered cells via endocytosis (**paper I**). I found, by comparing uptake in SH-SY5Y cells, CHO-K1 cells and NIH 3T3 fibroblasts, that the degree of cellular uptake of both Aβ(1-40) and Aβ(1-42) depends on the cell type's intrinsic endocytic rate (which I measured using the fluid phase marker dextran 10 kDa [205], as discussed in section 4.2.1) (Figure 35). This suggests that uptake occurs via generic endocytic mechanisms existing in many different cell types [222]. Further, this is interesting in relation to findings by Friedrich et al. [105] that cell types with high endocytic activity are most efficient in seeding the *in vitro* formation of plaques; a study that supports the idea that intracellular accumulation via endocytosis is directly important for pathological plaque deposition. In this context, it could be mentioned that, from a technical perspective, it would be reasonable to choose to perform further studies with one of the cell lines showing the highest Aβ intensity (and hence uptake) as this would aid detection. However, the SH-SY5Y cell model, although displaying the lowest endocytic rate, is commonly used in the field [223, 224], hence making direct comparisons to other work possible.

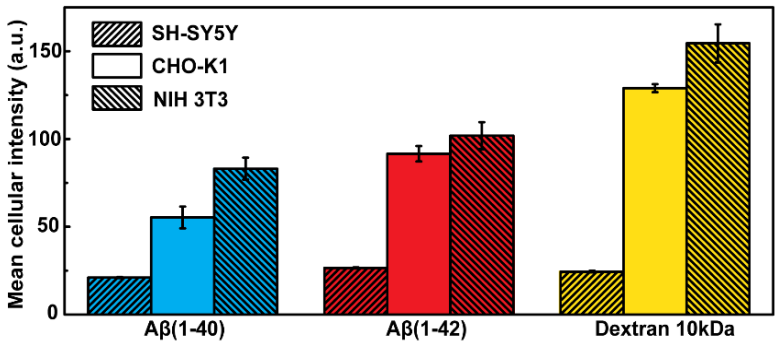

Figure 35. Aβ(1-40) and Aβ(1-42) uptake in cultured cells corresponds to endocytic activity, as measured by flow cytometry analysis of cellular uptake of Aβ peptides and dextran 10 kDa.

To further verify that the peptides enter cells via endocytic paths, I measured the uptake of Aβ at 4 °C and after depletion of ATP, both treatments known to block uptake via endocytosis [201, 202]. Incubation at 4 °C, however, not only blocks endocytosis but also changes the fluidity of the cell membrane [225] and I therefore decided to apply also the harsher treatment of ATP depletion. Both approaches resulted in highly reduced Aβ uptake, while cells were still viable and could recover after the inhibition, strengthening the evidence that uptake of Aβ(1-40) and Aβ(1-42) applied as monomers occurs via endocytosis. As a further characterization of the fate of endocytosed Aβ, I also measured the extent of the internalised peptide being transported to acidic organelles, such as lysosomes, by colocalization with LysoTracker. For both Aβ variants the co-localisation was in the range of 90 %, demonstrating efficient transport through and accumulation in the endolysosomal pathway. With time, the cells were, however, found to be able to clear the peptides; over 50 % of both isoforms were

cleared within the first 24 hours after exposure. This similarity suggests that the observed quantitative differences in intracellular accumulation of Aβ(1-40) and Aβ(1-42) do not originate from differences in clearing, but indeed from differences in uptake rate. These observations are also interesting in relation to observations on oligomeric forms of Aβ by Domert et al [226], demonstrating highly inefficient clearing of Aβ(1-42) but not of Aβ(1-40).

The following sections describe my work to map uptake paths and key molecular and mechanistic regulators in cellular internalisation of Aβ, based on the work contained in **paper I** and **IV**. It is divided in probing for clathrin-dependent and -independent paths, and sequentially in dynamin-dependent and -independent variants of clathrin-independent uptake.

4.3.1 Clathrin-mediated endocytosis

There is a range of membrane receptors suggested to be important for Aβ endocytosis, of which many are involved in clathrin-mediated endocytosis (CME), and for a review of this I refer to Lai and McLaurin [158]. In **paper I**, I therefore studied the role of CME in the endocytosis of Aβ(1-40) and Aβ(1-42) supplied as monomers. The uptake of both variants was reduced by treatment with chlorpromazine (CPZ) while no reductions in uptake were seen in cells transfected with AP180-C (which blocks CME) at conditions were the uptake of the well-known CME ligand transferrin (Trf) [135] was reduced (Figure 36). Instead, a small increase in Aβ(1-42), but not Aβ(1-40), uptake was seen in cells expressing AP180-C; this is interesting as it points to a difference in uptake mechanism of the two Aβ isoforms, and that a potential Aβ(1-42) specific path could be upregulated upon perturbation of CME. Such compensatory regulation has previously been seen upon perturbation of dynamin [227] and macropinocytosis [228]. Since expression of AP180-C did not result in reduction of Aβ uptake, the reductions with CPZ were considered as off-target effects of the inhibitor, which is rather toxic. This also points out the importance of not only relying on pharmacological inhibitors when mapping endocytic paths, but rather to use a combination of different approaches.

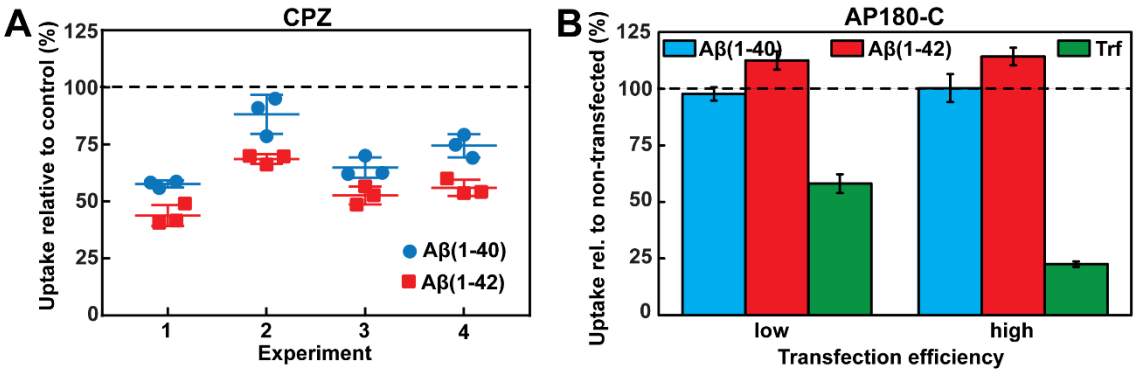

Figure 36. Evaluation of CME in uptake of Aβ(1-40) and Aβ(1-42). Uptake of Aβ in SH-SY5Y cells (A) treated with CPZ and (B) transfected with AP180-C. Analysis was performed by flow cytometry.

4.3.2 Clathrin-independent endocytosis

Clathrin-independent endocytosis (CIE) is a complex network of different paths and its characterization is complicated by the facts that some regulatory components (proteins) are involved in multiple paths, that there is a lack of specific ligands and, that the extent by which endocytic paths are used can differ vastly between cell types [222]. In this Thesis, I decided to divide CIE into two parts; dynamin-dependent (which CME also is) and -independent endocytosis. In section 4.3.2.1 I describe the role of dynamin-dependent CIE and survey for potential uptake via FEME, based on involvement of Rho GTPases. In section 4.3.2.2, I focus on dynamin-independent CIE, including studies on actin inhibitors, macropinocytosis, Arf6, as well as evaluation of GRAF1-dependent uptake via CLIC/GEEC based on modulations of plasma membrane tension. Here it can also be mentioned that even though I made a decision to make this division of endocytic paths, I would like these experiments to been seen more as a mapping type of approach. Based on the aggregating nature of the Aβ peptide, and in particular that it is very challenging to determine exactly which species are internalised (as they may form at the surface just prior to uptake) it is highly likely that several paths are involved in Aβ uptake. The range of different suggested receptors and uptake paths of Aβ reported in literature [158] supports this. Still, it is likely that certain components are more important for uptake than others.

4.3.2.1 Dynamin-dependent endocytosis

To test if internalisation of Aβ(1-40) and Aβ(1-42) is dependent on dynamin, I studied their uptake in cells exposed to the dynamin-inhibitor dynasore as well as in cells transfected with a DN variant of dynamin2; K44A (Figure 37, **paper I**). Similarly to in the experiments with CME in the previous section, I observed reductions with the pharmacological inhibitor (however, interestingly only for Aβ(1-42)), but not in cells transfected with K44A. Based on the results with K44A we concluded that the uptake of Aβ is likely dynamin-independent and that the reduction in Aβ(1-42) uptake in cells treated with dynasore could be an off-target effect of the inhibitor, as it has been shown to display dynamin-independent effects on cholesterol homeostasis, lipid rafts, membrane ruffling and actin (see Preta et al [229]). Independent of this, the difference in uptake between Aβ(1-40) and Aβ(1-42) in dynasore-treated cells is interesting as it, again, points to differences in uptake paths(s) between the two isoforms.

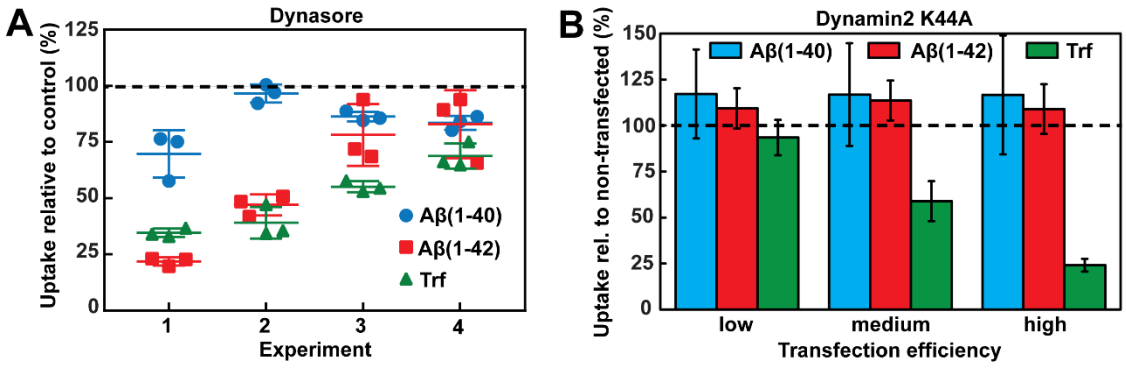

Figure 37. Evaluation of dynamin in uptake of Aβ(1-40) and Aβ(1-42). Uptake of Aβ in SH-SY5Y cells (A) treated with dynasore and (B) transfected with dynamin2 K44A. Analysis was performed by flow cytometry.

A recently described dynamin-dependent uptake mechanism is FEME [145]. This path is inhibited by inhibition of the Rho GTPases Rac1 and RhoA, but activated upon Cdc42 inhibition. In **paper IV**, I studied how overexpression of DA, WT and DN variants of the Rho GTPases Cdc42, Rac1 and RhoA affected the uptake of Aβ(1-42) in SH-SY5Y cells. The results showed that uptake was sensitive to regulation of Cdc42 and RhoA (Figure 38), but not to Rac1. The observed pattern is not in agreement with uptake via FEME, as expected considering the dynamin-dependent nature of this pathway. The observed responses are, however, still valuable, considering the proposed roles of Rho GTPases in AD pathogenesis [144].

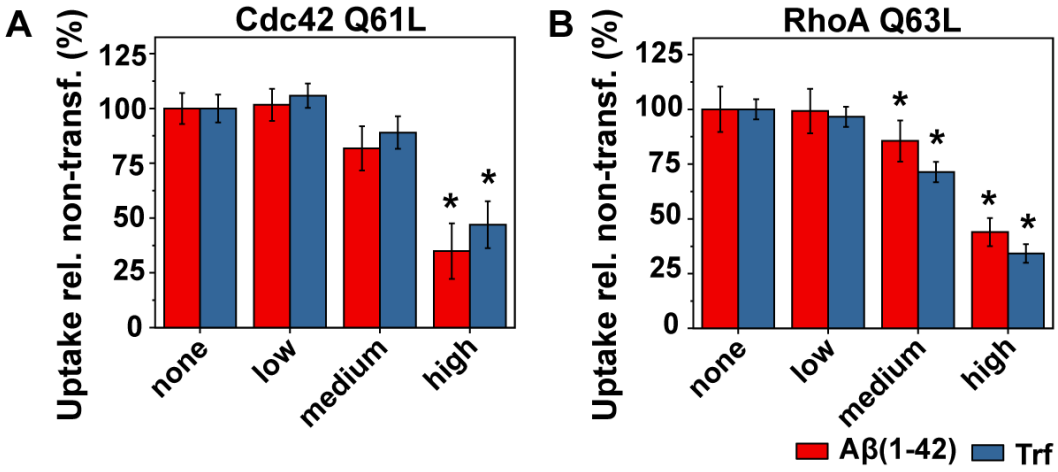

Figure 38. Involvement of DA variants of the Rho GTPases Cdc42 and RhoA in uptake of Aβ(1-42) and Trf in SH-SY5Y cells. Analysis was performed by flow cytometry.

A natural additional target to study in terms of dynamin-dependent endocytic pathways would be uptake via caveolae. However, neuroblastoma cells, as the SH-SY5Y cells used for the majority of the work presented in this Thesis, do not express caveolin [230], essential for formation of caveolae. In addition, recent findings have shown that caveolae are in fact not involved in endocytosis to the previously thought extent, but rather has a primary role in mechano-sensing [138]. Based on these two factors I did not include caveolae in my work.

4.3.2.2 Dynamin-independent endocytosis

Based on the regulatory effect of Rho GTPases in Aβ(1-42) uptake, actin polymerization appears to play an important role. Indeed, in **paper I,** I also showed that the uptake of both Aβ(1-40) and Aβ(1-42) is sensitive to perturbation of actin polymerization by exposure to the pharmacological inhibitors cytochalasin A and D (Figure 39). Interestingly, also here there are noteworthy differences in perturbation of Aβ(1-40) and Aβ(1-42) uptake upon treatment with the inhibitors, indicative of some differences in their uptake paths. Actin polymerization is important in both CME and CIE, but to various extents. Among others, actin is of high importance for the formation of membrane protrusions that drive dynamin-independent uptake via macropinocytosis [151]. I exposed cells to the macropinocytosis inhibitors IPA-3 and wortmannin and showed that the uptake of both Aβ(1-40) and Aβ(1-42) was reduced at conditions when Trf was not affected. Macropinocytosis thus seems to be important for Aβ

internalisation, although it is in this context surprising that I did not observe any influence of Rac1-inhibition, as this Rho GTPase is considered highly important in macropinocytosis. I also tried to use a large 70 kDa dextran as a macropinocytosis marker [205], but did not manage to get reliable and reproducible results. This problem has been noted previously; commercially available 70 kDa dextrans have been shown to contain contaminant free fluorescent dye and/or its degradation products [231], and there is also a risk that these large dextrans aggregate (R. Teasdale, personal communication); purification by size exclusion could perhaps have enabled their use.

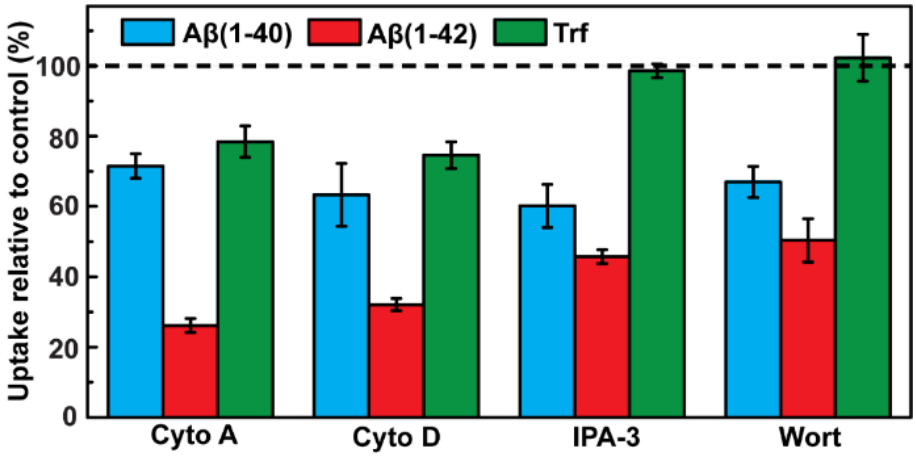

Figure 39.Uptake of Aβ(1-40), Aβ(1-42) and Trf in SH-SY5Y cells treated with the pharmacological inhibitors cytochalasin A and D, IPA-3 and wortmannin. The cells were anaysed by flow cytometry.

Uptake via Arf6 is a clathrin- and dynamin-independent endocytic path that, in addition, has been linked to the internalization of BACE1 [103], thereby being involved in the regulation of APP processing. I evaluated this path for Aβ(1-40) and Aβ(1-42) uptake (**paper I**), but no signs of involvement were observed. In addition, endocytosis via flotillin has previously been suggested to be important for endocytosis of APP, thereby directly involved in Aβ production [232, 233]. Recent reports have however indicated that flotillin does not directly participate in the endocytic process but rather plays an indirect role [138] and I therefore decided not to study potential uptake via this path in my work.

Another dynamin-independent uptake path is via CLIC/GEEC [148, 149], a path which has been shown to be dependent on the protein GRAF1 [147]. CLIC/GEEC is furthermore highly sensitive to reductions in membrane tension [132]. The influence of membrane tension on other endocytic paths are less established, but recent reports have indicated that it could be important in macropinocytosis as well [234]. In **paper IV**, I exposed SH-SY5Y cells to a series of experiments where the membrane tension of the cells was osmotically perturbed; confocal microscopy revealed that the uptake of both Aβ(1-42) and the fluid phase marker dextran 10 kDa was abolished in hypotonic medium, whereas the uptake of both were re-activated when the cells were brought back to isotonic medium (Figure 40). Both were found in vacuole-like dilations (VLDs) formed at the sudden reduction in membrane tension, although also additional small Aβ(1-42)-containing vesicles were observed. This indicates increased Aβ(1-42)-uptake via a specific upregulated endocytic path. I also studied the VLDs further by imaging them over

time; the structures were found to be highly dynamic displaying both tubulation and fission, resulting in their eventual clearance, in agreement with previously published literature [235].

I also quantified the uptake change observed in Figure 40 by flow cytometry (Figure 41). The results confirm a clear difference in behaviour of Aβ(1-42) from both Trf and dextran 10 kDa, further supporting the view that the peptide is not internalized through CME.

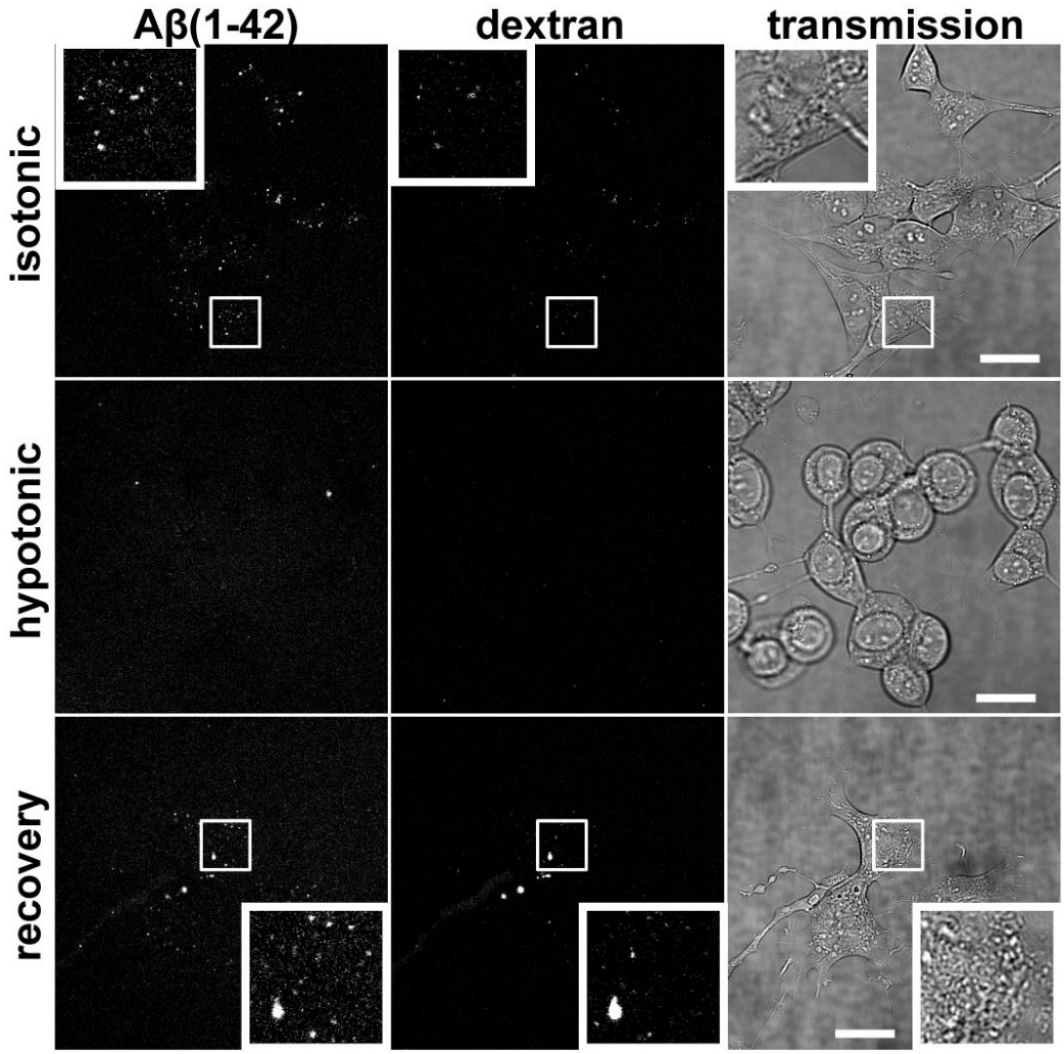

Figure 40. SH-SY5Y cells exposed to perturbations in membrane tension by changes in tonicity of the incubation medium. The cells were incubated with Aβ(1-42) and dextran 10 kDa and imaging was performed by confocal microscopy. The scalebar is 20 µm.

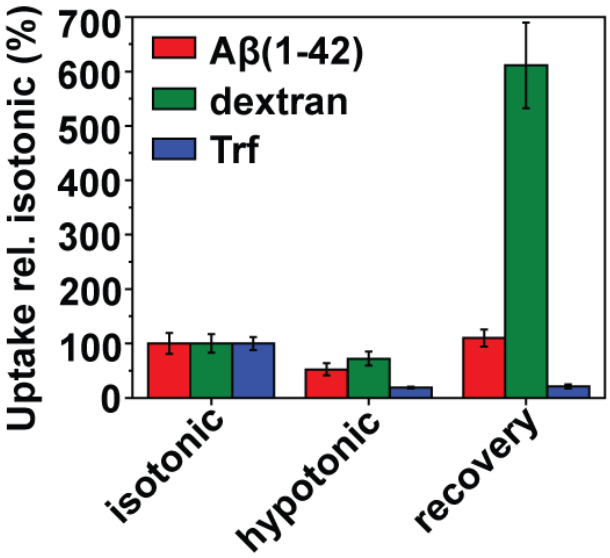

Figure 41. Flow cytometry-based quantification of Aβ(1-42), dextran 10 kDa and Trf uptake in cells exposed to perturbations of membrane tension.

The results from the above experiments could point to involvement of GRAF1-dependent CLIC/GEEC in Aβ(1-42) uptake. Also, the observed sensitivity to Cdc42-perturbtion could be consistent with this, although it is intriguing in this respect that I did not observe any influence of the DN variant of Cdc42 [148]. I therefore performed experiments to look for potential involvement of GRAF1 in Aβ(1-42) uptake (Figure 42). To do this I used a Flp-In T-REx HeLa cell line with inducible expression of GRAF1. When these cells overexpress DA Cdc42 Q61L, GRAF1 is accumulated in punctuative and tubular structures where ligands internalised though GRAF1-dependent CLIC/GEEC are also trapped [235]. Co-localisation of internalised Aβ(1-42) and these GRAF1-positive structures was not observed, suggesting that GRAF1 is not involved in Aβ(1-42) uptake. As uptake is highly dependent on membrane tension and the regulation of small GTPases, Aβ(1-42) may however be internalised through a GRAF1-independent uptake path of CLIC/GEEC type [132], or a novel uptake path not yet characterized.

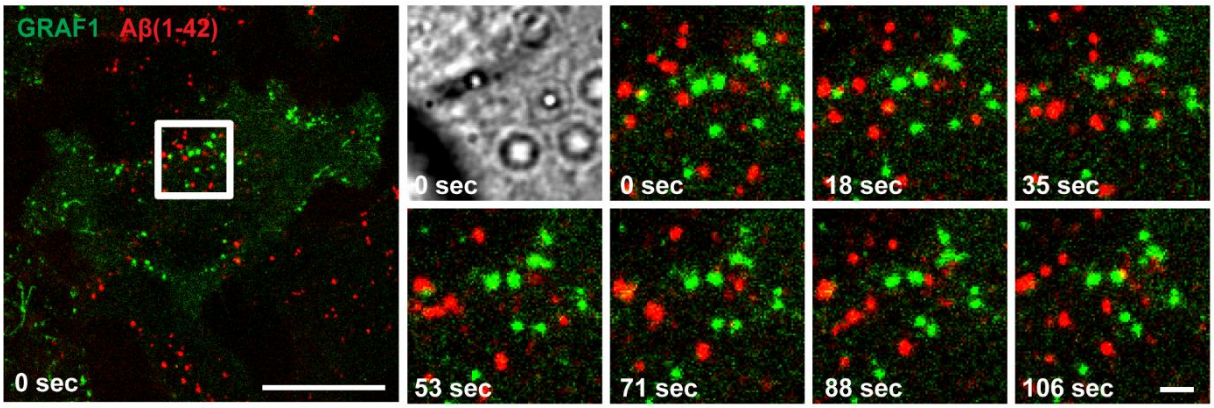

Figure 42. Confocal microscopy of GRAF1 Flp-In T-REx HeLa cells transfected with Cdc42 Q61L and incubated with Aβ(1-42). The scale bar in the left image is 20 µm and in the zoomed images 2 µm.

5 Concluding remarks & Outlook

5 Concluding remarks & Outlook

The focus of this Thesis has been to better understand intracellular accumulation of amyloidogenic proteins, in particular Aβ, following their uptake from the extracellular space. The quantitative foundation for this was laid in **paper I** where I showed that there is a robust difference in uptake between Aβ(1-40) and Aβ(1-42) of a factor two – interestingly appearing due to the presence of only two additional amino acids in the longer variant. This, in combination with the demonstrated large concentrating potential of endocytic uptake, is highly important for the understanding of intraneuronal Aβ accumulation, potentially elucidating the origins of why Aβ(1-42) is predominantly found in plaques.

The second study, **paper II**, was focused on quantitative aspects of fragmented forms of another protein, α-syn, and its relation to toxicity. The most important finding from this is that there is an inverse correlation of average fibril length and uptake, and that the species that are taken up most efficiently are also the most toxic. This, importantly, suggests an intracellular basis of toxicity. Based on the very inefficient uptake of the α-syn monomer, I did not follow up on α-syn monomers for comparisons with Aβ. It would, however, be interesting to complement with fibril fragmentation studies also on Aβ. Attempts were made, but as the Aβ fibrils displayed very different behaviour, further protocol development would be needed and was not applicable within the time frame of this Thesis work. Further, it would also be interesting to study the endocytic mechanism(s) of fragmented α-syn fibrils to address how size and structural assembly influence the distribution between different endocytic components and paths. In our experiments with α-syn, I did not observe internalization via CME, which was also not expected given the relatively large size cut-off at ~ 400 nm average length which would require the stiff fibrils to fold to be accommodated in clathrin-coated vesicles. Hence, further expansion on endocytic components and how this relates to the uptake of other amyloidogenic proteins and their respective aggregates, in particular Aβ, would be an important step forwards in this characterization. This could offer a means to relate aggregate structure to potential generic attributes that are of potential importance for pathogenesis, not only for better understanding of endolysosomal accumulation and toxicity, but also potentially elucidating the prion-like spread of amyloid aggregates.

In **paper III**, I followed up on the quantifications of Aβ uptake and elucidated details of the role of PGs. Most importantly, I showed that the PG-dependency in Aβ uptake has a temporal evolution, which has not been observed before, neither for Aβ nor any other cargo. From this, we proposed that PG-dependent local aggregation at the cell surface prior to uptake could be an important feature. This exemplifies that although the starting material and bulk processes are well characterized, it is very difficult to follow what occurs in the local cellular environment, for example in the proximity of cell membranes. It would be of interest to build on these findings, by studying more specifically what species are internalised, which could be possible by developing and applying further multi-dimensional imaging methods such as FRET. This also represents challenges in the even more complex *in vivo* setting, further complicating understanding of local protein structures and aggregation. However, this represents possibilities as well: by elucidating details of specific aggregated species, there is a possibility to target disease-relevant mechanisms with higher specificity. Therefore, the extensive work in the field,

elucidating details of cell interaction of amyloid oligomers and other aggregated species, together with technical developments enabling their detection, is of importance to understand Aβ pathogenesis; my work on monomeric Aβ adds an important piece to this complex puzzle.

In my studies of PG-dependency, I also realized that it was highly important to consider overall endocytic rate of the cells, since I, at early timepoints, observed a quantitative difference in Aβ uptake between the two lines, that in fact merely reflected the difference in endocytic rate of the PG-containing and PG-deficient cells. Based on this, I developed a novel approach for data compensation, which implies that several studies on PG-dependent uptake where the quantitative difference between cell lines is low, may in fact need to be re-evaluated. Within this Thesis, I have not further mapped if certain endocytic paths are more affected than others by the absence of cell-surface PGs, but this would be interesting and provide important additional information to the still immature understanding of how PGs mechanistically engage in various forms of endocytosis.

With its first part in **paper I**, and built-upon in **paper IV**, I have also examined how various endocytic paths and components are related to Aβ endocytosis. Interestingly, these experiments show that uptake of both Aβ(1-40) and Aβ(1-42) is independent of clathrin, despite that a large number of suggested Aβ-binding receptors are taken up via CME. Further, I found that blocking CME resulted in an upregulation in Aβ(1-42) uptake, but not Aβ(1-40). This could indicate compensatory upregulation of an Aβ(1-42)-specific path. Responsive differences between the two isoforms were observed also with other perturbations, suggesting that Aβ(1-40) and Aβ(1-42), despite their highly similar sequence, may take, at least partially, different endocytic routes. If Aβ(1-42) enters cells via an endocytic path not accessible to Aβ(1-40), this could help explain the quantitative difference in accumulation between the two, especially given that I did not observe any difference in the rate of their clearance. Both Aβ variants appeared to be independent of dynamin, but displayed sensitivity to perturbation of actin polymerization, as seen with both pharmacological inhibitors and regulation of Rho GTPases. This, in combination with pharmacological inhibition of macropinocytosis, suggests that uptake via macropinocytosis or a similar mechanism is important. Aβ(1-42) uptake was further shown to be sensitive to changes in membrane tension, indicating potential involvement of the CLIC/GEEC pathway, although seemingly GRAF1-independent. Taken together, this points to that Aβ(1-42) is taken up via a novel type of mechanism that is highly dependent on membrane tension and regulatory control of small GTPases in a process that appears to be similar to CLIC or macropinocytosis, but that involve yet uncharacterized molecular players. It would be intriguing to further examine the involvement of endocytic components by studying the responses in additional cell types, among these primary neurons, as difference in number and incidence of endocytic paths could facilitate understanding of Aβ uptake and accumulation.

The work presented in this Thesis has focused on the amyloid protein uptake event, as well as factors that influence quantitative aspects of this. In terms of intracellular Aβ and its relation to pathology, a natural continuation of this project would be to examine the intracellular fate of these species further, by examining their intracellular trafficking. Such attempts were initiated, based on overexpression of fluorescently labelled vesicle-specific markers and followed by time lapse imaging and analysis of vesicle movement, but further development is needed. Also, how amyloidogenic proteins potentially influence their respective vesicular carriers, as well as general processes of intracellular trafficking, are interesting lines of research to take as a continuation of the work presented in this Thesis.

6 Acknowledgements

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7 References

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